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Culture-independent detection and quantification of *Fusarium circinatum* in a pine-producing seedling nursery

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The primary symptoms associated with *Fusarium circinatum* infection in pine seedling nurseries are root and collar rot, shoot and tip die-back and seedling mortality. Management of this pathogen in nurseries usually involves the integration of various strategies relating to sanitation, insect control and fungicide treatment. The overall goal of this study was to use quantitative real-time PCR (qPCR) to detect and quantify the airborne inoculum of *F. circinatum* in a commercial pine seedling nursery. For this purpose, an existing qPCR method was optimised and evaluated for its efficacy to quantify and monitor airborne conidia over a one-year period. Results showed that *F. circinatum* occurred at relatively low levels in the nursery throughout the year and that its distribution was spatially sporadic. The data suggest that standard nursery sanitation practices in the test nursery maintained the airborne inoculum of *F. circinatum* at low levels. The uneven distribution of infection also suggests that airborne inoculum does not represent the primary source of inoculum for the *F. circinatum*-associated seedling disease.

Keywords: *Fusarium circinatum*, nursery disease, pitch canker, quantitative real-time PCR, spore load

Online supplementary material: Summaries of spore concentrations and weather data are available as online supplementary material at http://dx.doi.org/10.2989/20702620.2014.899058.

Introduction

Fusarium circinatum Nirenberg and O'Donnell emend. Britz, Coutinho, Wingfield and Marasas is the causal agent of pitch canker on *Pinus* spp. (reviewed by Wingfield et al. 2008). This pathogen has a worldwide distribution including the USA (Hepting and Roth 1953), Haiti (Hepting and Roth 1953), Japan (Muramoto and Dwinell 1990), Korea (Lee et al. 2000; Woo et al. 2010), Mexico (Britz et al. 2001), Chile (Wingfield et al. 2002), Spain (Landeras et al. 2005), France (EPPO 2006, 2010), Italy (Carlucci et al. 2007), Uruguay (Alonso and Bettucci 2009), Portugal (Bragança et al. 2009) and Colombia (Steenkamp et al. 2012). In South Africa, *F. circinatum* was first discovered in 1990 causing a serious disease of *Pinus patula* seedlings (Viljoen et al. 1994), and has since been recorded in almost all commercial pine seedling nurseries in the country (Wingfield et al. 2008). The symptoms caused by the pitch canker fungus in natural *Pinus* stands or plantations differ markedly from those observed in seedling nurseries (Wingfield et al. 2008). In the nursery, the pathogen infects seedlings, which leads to root and collar rot, shoot and tip die-back and ultimately seedling mortality, which is different to the branch die-back and stem cankers found in the field.

Management of *F. circinatum* infection in seedling nurseries is complex and usually includes the integration of various strategies such as sanitation (water, growth media and seedling containers), insect control (Hurley et al. 2007) and fungicide applications (Wingfield et al. 2008). Currently, very little is known regarding the abundance of the inoculum in nurseries and particularly not in terms of airborne inoculum. There is, however, a possibility that the conidia of *F. circinatum* can be distributed by the irrigation system. Rossi et al. (2002) studied the distribution of airborne *Fusarium* conidia in naturally infected wheat fields and concluded that large water drops will allow for short-distance dispersal of conidia. However, small water drops can become airborne and thus allow for conidia to be dispersed over longer distances. Airborne *Fusarium* conidia have also been reported within maize and sorghum fields (Inch et al. 2005; Funnell-Harris 2011; Donat et al. 2012).

Various spore trapping techniques have been used to detect airborne conidia (Fraedrich and Witcher 1982; Correll et al. 1991). A common approach has been to use culture-based trapping methods but these have the disadvantage of rapid desiccation of the growth medium, and inefficient quantification via fungal colony counts. Another important limitation of these culture-dependent approaches is the general lack of diagnostic characteristics for differentiating *F. circinatum* from other *Fusarium* species that contaminate the trap media (Leslie and Summerell 2006). Therefore, methods allowing direct detection and quantification of

the pathogen, without the need for culturing (i.e. culture-independent methods), are likely to provide more accurate estimations of the inoculum load that occurs in a particular environment, especially in seedling nurseries where multiple *Fusarium* species are likely to occur.

Most of the culture-independent methods that have been developed to detect and quantify microorganisms rely on the use of PCR and diagnostic DNA sequence information (Giraffa and Neviani 2001). Three DNA-based approaches are currently available to identify *F. circinatum*: (1) TEF-barcode analysis (Geiser et al. 2004); (2) *H3* PCR-restriction fragment length polymorphism (RFLP) (Steenkamp et al. 1999); and (3) PCR with *F. circinatum*-specific oligonucleotide probes or primers (Schweigkofler et al. 2004; Ioos et al. 2009; Dreaden et al. 2012). Of these, PCR-based methods using specific probes and primers allow for the simultaneous detection and quantification of the pathogen, especially when combined with quantitative real-time PCR (qPCR). Due to their sensitivity, these methods can be used to detect and quantify *F. circinatum* contamination in seed lots (Ioos et al. 2009; Dreaden et al. 2012) or to detect the pathogen in infected plant material (Schweigkofler et al. 2004; Ramsfield et al. 2008). These qPCR-based methods can also be used to obtain epidemiological data by, for example, quantifying the airborne conidia of *F. circinatum* associated with pitch canker outbreaks (Schweigkofler et al. 2004; Garbelotto et al. 2008).

The aim of this study was to use qPCR-based methods to detect and quantify the airborne conidia of *F. circinatum* in a commercial forestry seedling nursery in which the pathogen is known to be well established. To achieve this goal, the qPCR method described by Schweigkofler et al. (2004) was used to study *F. circinatum* spore loads in the nursery where *F. circinatum* was first detected in South Africa (Viljoen et al. 1994) during the course of one year. The specific aims were (1) to optimise the qPCR method to allow specific and sensitive detection of the reproductive propagules of *F. circinatum* and (2) to evaluate the use of this method to quantify and monitor airborne inoculum in the nursery environment.

Materials and methods

Fungal isolates

To optimise the qPCR method, two *F. circinatum* isolates (FCC2245 and FCC2265), as well as *F. subglutinans* (Wollenweber and Reinking) Nelson, Toussoun and Marasas (FCC992), *F. proliferatum* (Matsushima) Nirenberg (FCC3300) and four isolates of *F. oxysporum* Schechtendahl emend. Snyder and Hansen (CMWF1014, CMWF1041, CMWF911 and CMWF927) were used. These were grown on half-strength potato dextrose agar (PDA) medium (20 g l^{-1} PDA, 15 g l^{-1} agar; Biolab Diagnostics, Wadeville, South Africa) for 10 d at 25 °C. During the optimisation process, the two *F. circinatum* isolates were used to determine the sensitivity and repeatability of the protocol, while the *F. subglutinans*, *F. proliferatum* and *F. oxysporum* isolates were included to evaluate its specificity.

Spore suspensions for the two *F. circinatum* isolates were prepared using sterile distilled water to wash spores from the mycelium on the growth medium, followed by filtration through cheese cloth. Spore concentration was measured with a haemocytometer and a dilution series of 10^1 to 10^6 spores ml^{-1} (with increments of 10^1 spores ml^{-1}) was prepared for each sample. Of these spore suspensions, 3 ml were then spread across filter paper discs (Whatman No. 1, 70 mm diameter, Merck Laboratory Suppliers (Pty) Ltd, Durban, South Africa) and left to air dry before being used in subsequent experiments.

DNA extractions and qPCR optimisation

In all cases, a quarter of each filter paper spore trap (with surface area of 1.92×10^{-3} m^2) was used for DNA extraction using Chelex® 100 chelating resin (molecular biology grade, Bio-Rad Laboratories (Pty) Ltd, Johannesburg, South Africa). This method involved application of 1 ml of a 10% resin solution (prepared using the manufacturer's protocols) to a sterile 2 ml centrifuge tube containing the filter paper that had been cut into smaller pieces using sterile scissors, followed by mixing, incubation at 95 °C for 20 min and centrifugation at 10 000 $\times g$ for 1 min. In this way spores on the filter paper were disrupted in the presence of the Chelex resin that binds proteins and other components. The aqueous phase containing the fungal DNA was then used directly in PCR (Walsh et al. 1991; Möhlenhoff et al. 2001). DNA was also extracted from 10-day-old cultures of the *F. subglutinans*, *F. proliferatum* and *F. oxysporum* isolates as previously described (Groenewald et al. 2006).

Quantitative real-time PCR was performed on a Bio-Rad CFX 96 Real-Time apparatus. To determine the optimum conditions for amplification, qPCR was performed with the SsoFast™ EvaGreen® supermix (Bio-Rad) using a 0.5–5 μl range of starting DNA, a 0.2–1 μM primer (CIRC1A and CIRC4A) (Schweigkofler et al. 2004) and annealing temperatures of 60–65 °C. In each qPCR at a specific temperature, three amplifications were performed with each of the different combinations of starting DNA and primer concentrations. Where specificity of the qPCR method was evaluated, similar reaction conditions were used, but these included approximately 1 ng μl^{-1} DNA extracted from the three other *Fusarium* species. For the qPCR specificity tests, qPCR was also repeated at least three times. With the exception of annealing temperature, reaction conditions were retained as those recommended by Bio-Rad and were as follows: 95 °C for 10 min, followed by 44 cycles of 95 °C for 10 s, annealing of 60–65 °C for 15 s and 72 °C for 30 s. The amplification was completed by melt curve analysis of 95 °C for 1 min, 40 °C for 1 min and 65 °C to 95 °C with a 0.1 °C increment.

qPCR controls

In order to comply with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, various qPCR controls were included to test the reliability of the results (Bustin et al. 2009). To confirm that the qPCR fragment represented DNA sequences of *F. circinatum*, a number of the qPCR amplification products were subjected to agarose gel electrophoresis (Sambrook et al. 1989) and subsequent sequencing using the original primers (Schweigkofler et al. 2004). This qPCR confirmation step was included to ensure that the observed melt-curve peak for *F. circinatum* corresponds to its DNA sequences and not to those of the

non-target species. The non-target control species included were *F. subglutinans*, *F. proliferatum* and *F. oxysporum*, of which the latter two are common in the South African pine nursery environment, whereas *F. subglutinans* is a close relative of and morphologically similar to *F. circinatum* (Leslie and Summerell 2006).

To test the repeatability of the optimised qPCR method, samples positively identified as containing *F. circinatum* from different spore concentrations and batches in the test nursery (see below) were randomly selected and again subjected to two additional qPCR runs. In addition, 10 small blocks (10 mm × 10 mm) cut from all the filter paper disc traps of weeks 2 and 28 (see below) were placed onto *Fusarium* selective medium (Leslie and Summerell 2006) and incubated for 5–10 d at 25 °C. The presence of *F. circinatum* in these samples was then confirmed by microscopically examining the cultures and by subjecting them to the standard DNA-based diagnostic procedure, which involves the use of *F. circinatum*-specific primers and agarose gel electrophoresis for visualisation of the amplicons (Schweigkofler et al. 2004).

To assess the effect that false negatives (i.e. non-detection of *F. circinatum* in a sample actually containing it) might have on the results, DNA quality controls were included using DNA obtained from three poor-quality filter paper discs (e.g. showing evidence of weather or mechanical damage or that were soiled). These quality-control DNA samples were used in qPCR that also included the DNA extracted from the filter paper discs, which were spiked in the laboratory with different concentrations of *F. circinatum* spores.

Nursery inoculum levels

This study was conducted at the Sappi Ngodwana seedling nursery (25°35′4.74″ S, 30°38′18.05″ E). Whatman No. 1 filter paper discs were used as the solid surface onto which airborne conidia of the pathogen could be deposited and become trapped. Sample traps were collected from 27 positions in the nursery from November 2008 until October 2009 at two-weekly intervals. Each collection was referred to as a batch and batches were chosen to include positions near seedling tray tables, near the pine hedge bank area on the periphery of the nursery as well as from three surrounding areas somewhat distant from the nursery (Figure 1). At each position, filter paper spore traps were placed at three heights: 20 cm above ground level, at the seedling tray height (85 cm) and 200 cm above ground level resulting in a total of 81 sampling points. Each filter paper was moistened with 4× TE buffer (40 mM Tris-Cl [pH 7.5], 4 mM EDTA [pH 8.0]) prior to placement at the various positions and both sides of the filter papers were exposed to air. The filter paper samples were collected and replaced with fresh traps at two-week intervals and stored at 4 °C until they could be processed using the previously described method for DNA extraction and the optimised qPCR methodology, as well as a negative water control for each batch of samples.

The qPCR results were summarised to indicate trapping frequency (proportion of filter paper discs on which *F. circinatum* was detected) and to predict inoculum concentrations in the nursery during the study period. One-way analysis of variance (ANOVA) was used to determine if trapping frequency and inoculum concentration differed significantly between batches, overall positions and trapping height within the nursery. Tukey's test was used to compute significant differences among means ($P < 0.05$).

Results

DNA extractions and qPCR optimisation

Optimisation of the qPCR method included consideration of both its specificity and sensitivity to detect *F. circinatum*. Except for the lowest dilution (i.e. 38 × 10^2 spores m^{-2}), all of the tested annealing temperatures (60–65 °C) allowed for the detection of *F. circinatum* inoculum across

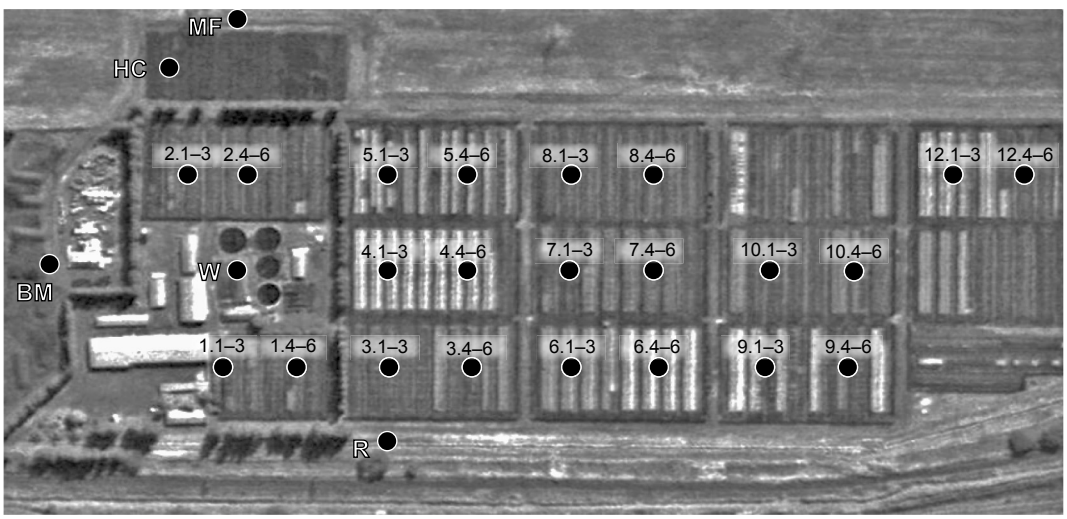

Figure 1: Aerial representation of the Ngodwana nursery showing the position of 81 *F. circinatum* trapping sites. These included 22 randomly selected sites next to nursery tray tables (1–22) and single sites in the hedge camp (HC), next to the water reservoirs (W), as well as in the surrounding area near the bark mill (BM), maize field (MF) and railway line (R). At each of these sites, three spore traps were placed at three different heights: 20 cm above ground level, at the seedling tray height (85 cm) and 200 cm above ground level

the concentration range tested (i.e. 38×10^3 to 38×10^7 spores m^{-2}). At the lower end of the temperature range, the method was less specific and also allowed amplification of DNA extracted from *F. oxysporum*, *F. subglutinans* and *F. proliferatum*. With an annealing temperature of 65 °C, the method was most specific and only allowed amplification of DNA extracted from *F. circinatum*, but it did not yield consistent results at the lower spore concentrations.

To reduce the risks of false positive results, an annealing temperature of 65 °C was used in the nursery spore load study and this was also used to construct a standard curve against which to quantify the spore concentrations. The qPCRs consisted of 0.5 µl starting DNA extract, 0.1 µM primer mix and 10 µl of SsoFast™ EvaGreen® supermix made up to a total volume of 20 µl with distilled water. Although use of additional DNA and primers allowed for amplification of the lowest dilution tested (i.e. 38×10^2 spores m^{-2}), these gave rise to primer-dimers that significantly influenced the accuracy of DNA quantification with qPCR. At the optimised conditions, the qPCR thus allowed specific and quantitative detection of *F. circinatum* at spore concentrations higher than 38×10^2 spores m^{-2}. At the optimised conditions the efficiency of all qPCRs ranged from 90% to 110%.

qPCR controls

Electrophoretic and DNA sequence analyses of various randomly selected qPCR amplicons showed that the sequence of the diagnostic 360 bp fragment generated with the CIRC1A and CIRCA primers (results not shown) corresponded to that known for *F. circinatum* (Schweigkofler et al. 2004).

Repetition of the qPCR assay on randomly selected samples that were positively identified as containing *F. circinatum* yielded varying results. This variation was particularly pronounced at the lower spore concentrations ($<38 \times 10^2$ spores m^{-2}) where *F. circinatum* spores were not always detected in subsequent runs. The high spore concentration samples sometimes also produced varying results, although this was only with regards to the specific concentration detected and not the presence and/or absence of the fungus (results not shown). These varying results, however, were always linked to the quality of the filter paper tested (see below).

The qPCR results corresponded with the culture-dependent detection of *F. circinatum* on the filter papers of weeks 2 and 28. The results of both detection approaches showed that only a few samples from week 2 contained detectable levels of *F. circinatum* (i.e. five samples based on culturing and seven samples based on qPCR). In the case of the samples from week 28, culturing suggested that 18 of 81 filter paper samples harboured *F. circinatum*, while qPCR indicated that 27 filter paper samples contained the fungus. Such differences were likely due to the limitations associated with the culture-dependent approach, as only 10 randomly selected blocks of the remaining three-quarters of individual filter paper discs were used from which to identify the fungus. Due to this approach and the fact that spores are unlikely to be deposited equally across the surface of the paper discs, quantification using qPCR did not exactly match the responses obtained with culturing, although there was agreement in the overall trends observed.

For the DNA quality controls, *F. circinatum* was detected only on two of the three poor-quality filter paper samples. This was despite the fact that the extracted DNA from poor-quality filter paper samples were spiked with DNA that was extracted from good-quality filter papers containing high concentrations of *F. circinatum* spores. The results therefore suggest that qPCR will not allow for the accurate detection of *F. circinatum* trapped on poor-quality filter paper samples (that may have been compromised by adverse environmental conditions), even though spores of this fungus may be present on the sample.

Nursery inoculum levels

Fusarium circinatum was detected in the Ngodwana nursery throughout the one-year period of the study. In each two-weekly sample batch, the fungus was detected on approximately six of 81 filter paper traps (7.4%). The only exceptions were weeks 26, 38, 42 and 50, where the fungus was not detected, and weeks 4 and 14, for which no filter paper traps were available (Figure 2, Supplementary Table 1).

Overall, the trapping frequency (proportion of filter paper discs on which *F. circinatum* was detected) was low with more than 90% of the 81 spore traps per batch not showing any detectable levels of the pathogen (Table 1, Figure 2). The trapping frequency was generally higher in the December to March period (i.e. weeks 6, 8, 10, 12, 16, 18 and 20) during which the pathogen was detected on 10 or more of the 81 filter traps per batch (Figure 2, Supplementary Table 2). The higher trapping frequency coincided with higher rainfall recorded for these months (Supplementary Figure 2), but there were some exceptions. The batch with the highest trapping frequency (22 filter paper discs) was from week 28 (11 May), while in batches preceding and following week 28, the pathogen was either not detected (i.e. week 26; 24 April) or *F. circinatum* was detected on only one filter paper trap (i.e. week 30; 25 May) (Supplementary Table 1).

The filter paper discs placed at 200 cm above ground level had the lowest trapping frequency, while those at table height (85 cm) had the highest trapping frequency for *F. circinatum* (Table 1). Of the traps collected at the 200 cm height positions, less than 4% had detectable levels of *F. circinatum,* compared to 10% at table height. Although the trapping frequencies at the 20 and 85 cm above-ground-level positions were not markedly different, these were significantly different ($P < 0.05$) from those recorded at the 200 cm above-ground-level positions (Table 1, Supplementary Table 2). Note, however, that the trapping frequencies recorded at the 20 cm above-ground-level positions probably represent underestimates because of the occurrence of false negatives. These are associated with the overall poor-quality of the filter paper discs placed in this position, which was predominantly due to the back splash of soil on to the paper traps during the routine irrigation of the seedling crops.

During the one-year period of study in the nursery, the number of spores captured on the filter paper traps ranged from 0 to 38×10^7 spores m^{-2} (rounded off and recorded in

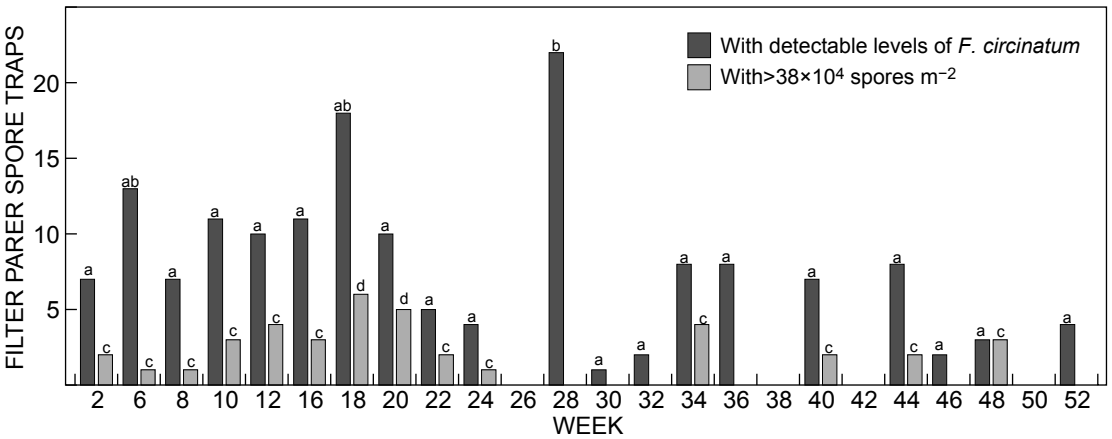

Figure 2: The number of filter paper spore traps from each two-weekly batch on which *F. circinatum* was positively identified (black bars; significantly different values [$P < 0.05$] are indicated by the letters 'a' and 'b' above the bars), as well as those on which spore concentrations of more than 38×10^4 spores m^{-2} were detected (grey bars; significantly different values [$P < 0.05$] are indicated by the letters 'c' and 'd' above the bars)

Table 1: *Fusarium circinatum* airborne spore trapping frequency and average concentration at different heights over a one-year period at the Ngodwana nursery

Spore trap height from ground (cm)	Trapping frequency (%)[a]	Average spore concentration per m^2 [b]
20 cm	8.4	10×10^3
85 cm	10.1	18×10^5
200 cm	3.7*	84×10^4
Combined data	7.4	

[a] The trapping frequency represents the proportion of samples in which *F. circinatum* was detected at a specific trapping position. The asterisk indicates the trapping frequency that was significantly differentiated from the rest ($P < 0.05$)

[b] The spore trap concentration average represents the total spore concentration observed over the total number of sites at a specific trapping position. No differences were detected among the averages at the 5% significance level

terms of the standards used). However, the spore concentrations on more than 98% of the samples were low and ranged from 0 to 38×10^3 spores m^{-2}. At each of the 27 collection sites in the nursery, the estimated number of trapped spores was higher on the filter paper discs that were positioned at table height (85 cm), but not significantly different compared to those at 20 cm or 200 cm above ground level (Table 1, Supplementary Table 2). Higher *F. circinatum* spore concentrations were recorded mostly during weeks 8–22 (December to March) (Figure 2, Supplementary Tables 1 and 2), for which higher trapping frequencies were also observed. However, trapping frequency did not necessarily correspond with the estimated spore concentrations, e.g. the highest trapping frequency was observed during week 28, but the concentration of spores in all 81 traps of week 28 ranged from 0 to 38×10^3 spores m^{-2}.

No obvious or statistically significant distribution pattern (i.e. spatially and temporally) was observed with respect to either trapping frequency or the spore concentrations detected (Supplementary Table 2). These estimates varied strikingly across the 27 test sites in the nursery, with distinct infection foci occurring and disappearing throughout the year. In some instances, the *F. circinatum* spore levels were concentrated at specific locations. For example, during week 2, higher spore loads were detected in blocks 17 and 19, which are all in close proximity. This was despite the fact that foci were usually absent at the same location in the sample batch from the following time interval (Supplementary Table 1). In some weeks, *Fusarium circinatum* was also detected in the surrounding areas somewhat distant from the nursery, where the seedlings were produced.

Discussion

Results of this study showed that in a South African seedling nursery where pine seedlings are propagated, inoculum of *F. circinatum* could be detected at much lower levels than those associated with pitch canker infections in-field (Schweigkofler et al. 2004; Garbelotto et al. 2008). Analyses conducted over a one-year period in a natural stand of *P. radiata* in California, displaying obvious symptoms of pitch canker, showed trapping frequencies greater than 25% and spore concentrations in the range of $5–10 \times 10^4$ spores m^{-2} or more (Garbelotto et al. 2008). In contrast, during our one-year nursery study, only 8% of the traps accumulated detectable levels of *F. circinatum* and, of these, less than 25% had spore counts comparable to those reported in California. Despite these low levels, *F. circinatum* was detected in the nursery throughout the study period. This showed that the management practices applied at the time of the study did not completely eliminate the pathogen from the nursery environment.

There was no obvious pattern in the spatial distribution of airborne *F. circinatum* inoculum. Sampling positions that were characterised by high spore counts for one collection period were not necessarily in close proximity to other sampling positions with high spore counts. The interpretation of this result is that the source of airborne inoculum was distinctly discontinuous in the nursery. This was most likely

due to isolated foci of seedling infection where sporodochia of *F. circinatum* occur at the bases of dying seedlings and from which conidia would be produced. The nursery in which this study was conducted has a sanitation procedure where symptomatic seedlings are removed (rogued) from the nursery daily (Mitchell et al. 2011). Thus, different and isolated infection foci would emerge over time and these would not relate to the fixed positions in which the traps were maintained in this study (Mitchell et al. 2011).

Despite occurring at relatively low levels throughout the year, the *F. circinatum* spore counts in the nursery varied over time and appeared to peak during certain months. This periodical variation in airborne spore counts could be ascribed to a number of factors. For example, during periods when predominantly low levels of spores were detected, the seedlings in the nursery were on average four-months-old or younger. These markedly smaller plants might therefore escape potential infection and hence contribute to the reduction in airborne inoculum. Other factors that could influence sporulation and spore deposition rates of *F. circinatum* are temperature and moisture availability (Schweigkofler et al. 2004). Previous studies in natural and planted stands of *P. radiata* in California showed that *F. circinatum* spore deposition rates and concentrations varied seasonally, but peaked during the cooler rainy season (Schweigkofler et al. 2004; Garbelotto et al. 2008). In this study peaks were observed during months with higher rainfall. Although these two factors would also be important for *F. circinatum* conidial development in the nursery, their effects would probably be more difficult to detect and quantify because the nursery environment is contained and more closely controlled than the open plantation situation.

Most of the filter paper spore traps analysed in this study revealed *F. circinatum* inoculum levels near or below the detection limit of the qPCR method used. The detection limit of qPCR is usually empirically determined and can vary greatly among different qPCR methodologies (Kennedy and Oswald 2011). Mavrodi et al. (2007) was able to detect *Pseudomonas fluorescens* from plant roots from a minimum of 4×10^6 colony forming units ml^{-1} and Selma et al. (2008) could detect *Aspergillus carbonarius* from wine grapes from a minimum of 1×10^3 conidia ml^{-1}. Based on the results of the present study and those reported by Schweigkofler et al. (2004), the reliable detection limit for *F. circinatum* using the qPCR method implemented here would be about 1×10^2 spores ml^{-1}. This is equivalent to the most prevalent concentration (38×10^3 spores ml^{-2}) detected in our study. The overall low spore counts detected in this study could thus explain some of the variation observed in repeat experiments.

A problem that arose in this study was the fact that filter paper traps became soiled with dust or contaminated with environmental impurities. The negative effect of these potential inhibiting compounds in PCR is widely recognised and considered as one of the most important obstacles associated with qPCR-based studies (Kennedy and Oswald 2011). In their study of *Entomophaga maimaiga* from a range of forest regions, Castrillo et al. (2007) showed that soil contaminants can inhibit qPCR, which is consistent with findings in the present study when DNA extracted from soiled filter paper discs was used in qPCR (i.e. false negatives). The presence of such inhibitors could have caused the markedly lower *F. circinatum* trapping frequencies and spore counts observed for the samples collected at the 20 cm above-ground-level positions. Castrillo et al. (2007) also showed that different soils can affect the accuracy of qPCR-based quantifications. The presence of such contaminants could also explain the variation in our repeat experiments for quantifying *F. circinatum* spore loads.

The discontinuous distribution and relatively low spore counts of *F. circinatum* observed in this study suggest that airborne conidia probably do not represent the primary source of inoculum for the frequent outbreaks of the seedling disease caused by this pathogen. This is in contrast to what is expected in forests and plantations where the fungus can be isolated from the aerial parts of the trees and commonly from the cones that are infected (Wingfield et al. 2008). Consequently, outbreaks of pitch canker in natural pine stands and plantations are associated with substantial aerial inoculum of the pathogen (Schweigkofler et al. 2004; Garbelotto et al. 2008). In the nursery environment, *F. circinatum* primarily infects the roots or root collars of seedlings (Barnard and Blakeslee 1980; Viljoen et al. 1994; Wingfield et al. 2008; Mitchell et al. 2011). With the implementation of routine nursery culling regimes, it is therefore unlikely that large airborne inoculum loads of the pathogen would develop from infected seedlings. Future research should thus explore other possible sources of inoculum, including growth media, seedling containers and irrigation water. Such studies will contribute to reducing the debilitating losses due to *F. circinatum*-associated seedling disease in South African commercial forestry nurseries.

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