

Development and evaluation of a real-time PCR seed lot screening method for *Fusarium circinatum*, causal agent of pitch canker disease

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Summary

Fusarium circinatum is a serious pathogen of *Pinus* spp. worldwide, causing pitch canker disease. *F. circinatum* can contaminate seeds both internally and externally and is readily disseminated via contaminated seed. Many countries require screening of pine seeds for *F. circinatum* before they can be imported. The currently accepted screening method is based on culturing the pathogen on a semi-selective medium and identifying it using morphological traits. This method is time-consuming and does not allow for accurate identification of the pathogen to the species level. A bulk DNA extraction and real-time PCR procedure to screen seeds for the presence of *F. circinatum* were developed in this study. The real-time PCR method resulted in the detection of *F. circinatum* in 5 of 6 commercial seed lots tested and has a lower detection limit of 1×10^{-5} ng of *F. circinatum* DNA per PCR. The culture-based method detected *Fusarium* spp. in four of six of the same seed lots. The real-time PCR method can be used to screen multiple seed lots in 2 days, whereas the culture-based method requires a minimum of 1–2 weeks. This new real-time PCR seed screening method allows for fast, sensitive and accurate screening and can be adapted to handle larger volumes of seeds.

1 Introduction

Pitch canker, caused by *Fusarium circinatum* Nirenberg & O'Donnell [*F. subglutinans* (Wollenweb and Reinking) Nelson, Toussoun, and Marasas f. sp. *pini* (teleomorph: *Gibberella circinata* Nirenberg and O'Donnell)], is a serious disease of *Pinus* spp. in many parts of the world (Brasier 2008; Stenlid et al. 2011). Because *F. circinatum* is readily transported in and on *Pinus* seeds, many countries require *Pinus* seeds to be screened for the presence of the pathogen before they can be imported (Anonymous 2009). Currently, the International Seed Testing Association (ISTA) seed screening method, referred to as blotter paper method in this paper, as applied at the United States Department of Agriculture Forest Service Resistance Screening Center relies on culturing the pathogen from seed on blotter paper infused with PCNB broth medium and identifying suspect colonies morphologically (Don 2002). This method does not allow for reliable identification of suspect colonies to the species level because observation of coiled hyphae, which are needed to identify colonies as *F. circinatum* (Nirenberg and O'Donnell 1998), is not required by this method and was not always observed during this study. This lack of certainty in identification at species level can result in false-positive results because of misidentification of suspect colonies. This method can also lead to false-negative results because many fungi can grow out of the seeds intermingling with or covering *F. circinatum* colonies. Additionally, this method is time-consuming; thus, quick screening of large numbers of seed lots is difficult because of the 1–2 or more weeks needed for fungal growth and hours of laboratory time needed to screen a seed lot. Because of these limitations, a new method of screening pine seeds for *F. circinatum* is needed that can provide high throughput at low cost with accurate results. Real-time PCR has the potential to improve the seed screening methodology allowing for more rapid, lower cost and more accurate seed screening (Schaad and Frederick 2002).

Two real-time PCR methods for detecting *F. circinatum* have been published. The first, by Schweigkofler et al. (2004), was used to measure the airborne conidia and not tested on seeds. The second method, developed by Ioos et al. (2009), was used to detect *F. circinatum* in seeds using a bio-enrichment step and extracting DNA from a subsample of the seeds; however, this method was not compared to the currently used screening method.

The objectives of this study were to (i) develop an improved real-time PCR screening method for *F. circinatum* in seeds; (ii) compare the detection results with the blotter paper-based method; and (iii) compare the cost and time needed for screening commercial slash pine (*P. elliotii* var. *elliotii*) seed lots with the blotter paper-based method and the new real-time PCR-based method.

2 Materials and methods

2.1 Seed lots

Samples from six commercially available seed lots of *Pinus elliotii* Engelm. var. *elliotii* consisting of approximately 5000 seeds each were used in the study. Six seed lots were used because this is the maximum number that the USDA Forest Service Resistance Screening Center (RSC) can process at one time during commercial screening (J. Bronson, personal Communication). The test seed lots were collected between 2003 and 2007 from three different orchard locations (3 from southern

Mississippi, 2 from southern Georgia and 1 from southern Louisiana). The seeds were stored dry at 4°C in our laboratory before they were processed.

2.2 Extraction of DNA

DNA was extracted from 400 seeds (approximately 15 g) with three replications per seed lot. A modified CTAB (hexadecyl trimethyl-ammonium bromide)-based DNA extraction method was used (Murray and Thompson 1980; Doyle and Doyle 1987; Carlson et al. 1991). For each lot, seeds were ground with liquid nitrogen using a mortar and pestle. The ground seeds were placed in 250-ml centrifuge bottles with 90 ml of nuclear extraction buffer (60 ml nuclear lysis buffer [200 mM Tris (tris(hydroxymethyl)aminomethane), 50 mM EDTA (ethylenediaminetetraacetic acid), 2 M NaCl, 2% (g/ml) CTAB, pH to 7.5 with HCl]), 40 ml water and 0.8 g Na bisulphate, 18 ml of 5% (g/ml) N-lauroylsarcosine and 10 µl RNase A (Thermo Scientific Epsom, Surrey, UK), which were then inverted 5–6 times to mix. The bottles were incubated at 65°C for 20 min, then chilled on ice to room temperature. An equal volume of 25 : 24 : 1 phenol (pH 7.9)–chloroform–iso-amyl alcohol was added and mixed by inverting 30 times, then centrifuged for 15 min at 15 300 rcf (relative centrifugal force). The supernatant was transferred to new 250-ml bottles where an equal volume of chloroform was added and inverted 30 times to mix before being centrifuged for 15 min at 15 300 rcf. The supernatants were transferred to new 250-ml bottles and isopropanol (0.6 times the volume of supernatant) was added, inverted 5–6 times to mix and centrifuged for 15 min at 3220 rcf. The DNA pellets were left at the bottom of the bottles, while the supernatants were poured off and discarded. The DNA pellets were washed with 2 ml of 75% ethanol and centrifuged at 3220 rcf for 5 min. The ethanol wash was discarded, and the DNA pellets were left to air-dry before being redissolved in 2 ml of TE buffer (10 mM Tris, 1 mM EDTA, bring to pH 8 with HCl). All DNA extractions were visualized on a 1.5% (g/ml) agarose gel to check genomic DNA quality.

Each DNA extraction was purified using PVPP (polyvinylpyrrolidone) columns adapted from Schena and Ippolito (2003). The bottoms of the spin columns (Fisher Scientific Inc., Suwanee, GA, USA) were removed, approximately 40 mg of PVPP was placed inside the spin columns, and the columns were placed in collection tubes. 400 µl of water was added to the columns and centrifuged at 3380 rcf for 3 min; this was repeated with 200 µl of water to pack the PVPP in the columns. The columns were placed in new 1.5-ml collection tubes; 50 µl of DNA/TE buffer solution was added and centrifuged at 3380 rcf for 3 min. The flow-through containing the purified DNA was retained.

The concentration of the purified DNA was determined using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA), and all seed DNA extractions were diluted with water to 50 ng/µl for use with real-time PCR.

DNA was extracted from a pathogenicity-tested and confirmed *F. circinatum* isolate (isolate 5, Fig. 3) obtained from Seed lot 2 during the blotter paper seed screening using an extraction method developed by Justesen et al. 2002 without freeze-drying the spores or the proteinase K treatments. This DNA was used to construct a serial dilution (1, 0.1, 0.01, 0.001, 1×10^{-4} , 1×10^{-5} and 1×10^{-6} ng/µl), which was used to make the standard curve, to find the detection limit and as a positive control in the testing of both real-time PCR methods.

2.3 Real-Time PCR protocol testing and optimization

Two existing real-time PCR protocols used for detecting *F. circinatum* in different settings were tested. The first was developed by Ios et al. (2009) and uses a dual-labelled probe as the detection method. The second real-time PCR protocol was developed by Schweigkofler et al. (2004) and uses SYBR Green I® (Molecular Probes Inc., Eugene, OR, USA) as the detection method. Methods were followed according to the literature cited.

2.4 Dual-labelled probe real-time PCR

The protocol based on the dual-labelled probe FCIR-P, and primers FCIR-F and FCIR-R (Ios et al. 2009) (Table 1), was tested using *F. circinatum* DNA extracted from a pure culture and DNA extracted from *F. circinatum* contaminated seeds. Multiple amplicons that were longer than expected (250–1000 bp) were observed when the real-time PCR products were analysed on a

Table 1. Primers and dual-labelled probe used in the study.

Name	Sequence (5'-3') ¹	Product size (bp)
CIRC1A ²	CTTGGCTCGAGAAGGG	360
CIRC4A ²	ACCTACCTACACCTTCACT	360
CIRC1L ³	CTTGGCTCGAGAAGGGACA	360
CIRC4L ³	ACCTACCTACACCTTCACTTT	360
FCIR-F ⁴	TCGATGTGTCGTCTCTGGAC	146
FCIR-R ⁴	CGATCCTCAAATCGACCAAGA	146
FCIR-P ⁴	6-FAM/CGAGTCTGGCGGGACTTTGTGC/3BHQ1	146

¹6-FAM = 6-carboxyfluorescein, BHQ1 = Black Hole Quencher 1, registered trademark of Biosearch Technologies Inc., Novato, CA, USA.
²Schweigkofler et al. (2004).
³Based on Schweigkofler et al. (2004).
⁴Ios et al. (2009).

1.5% (g/ml) agarose gel, Fig. 1, while using the published protocol (Ioos et al. 2009). A range of MgCl₂ concentrations, 2–6 mM, and two commercial Taq polymerases were used to eliminate the additional amplicons; however, they persisted. Use of this method was discontinued in the study because of the presence of additional amplicons.

2.5 SYBR green real-time PCR

The SYBR Green I[®]-based real-time PCR protocol, using the primers CIRC1A and CIRC4A (Table 1), was developed by Schweigkofler et al. (2004) to detect and quantify airborne conidia of *F. circinatum*. The protocol resulted in consistent problems with primer dimer formation, so several modifications were employed in an attempt to improve the performance. Three Taq polymerases, differing Mg concentrations (3–4 mM) and alterations to the thermocycling protocol (annealing temperature range of 63–67°C) were tried, but none of the modifications eliminated the primer dimer; thus, a modification of this method was employed.

The software program, Primer3 (Rozen and Skaletsky 2000), was used to assess the self-compatibility of the primers and the intergenic spacer (IGS) region of the rDNA of *F. circinatum*. By adding three bases to the 3' end of primer CIRC1A and two bases to the 3' end of primer CIRC4A, the primers were more compatible with each other (Table 1). Using the modified primers, renamed CIRC1L and CIRC4L, a single amplicon, without primer dimer, was consistently produced.

For SYBR Green real-time PCR, each reaction consisted of 2.5 µl of 10× PCR buffer, 2.5 µl of 2 mM deoxynucleoside triphosphate, 1 µl of 10 mM CIRC1L, 1 µl of 10 mM CIRC4L, 0.4 µl of 10× SYBR Green I[®] solution (Lonza Inc., Rockland, ME, USA), 0.1 µl Platinum Taq polymerase (Invitrogen Corp., Carlsbad, CA, USA), 15.45 µl H₂O and 1 µl of DNA template. All of the primers and probes used in this study were synthesized by Integrated DNA Technologies Inc. (Skokie, IL, USA). The optimal annealing temperature of 66°C was determined by testing the range between 63 to 72°C (data not shown). The thermocycling profile consisted of denaturation at 95°C for 180 s, followed by 45 cycles of 95°C for 35 s, 66°C for 55 s and 72°C for 50 s. A melting curve analysis was performed upon completion. All real-time PCR used in this experiment were replicated in triplicate with the *F. circinatum* serial dilution and a non-template negative control was present for every seed screening run using an Eppendorf Mastercycler ep Realplex (Eppendorf Inc., Hauppauge, NY, USA).

Taxon specificity of the modified primers (CIRC1L, CIRC4L) was determined by testing the DNA from 10 *Fusarium* spp. The *Fusarium* spp. were chosen because of their taxonomic proximity to *F. circinatum*, similarity to primer sequences or association with pine seeds. The primers CIRC1L and CIRC4L were tested using the DNA from *F. succisae* NRRL 13613, *F. bulbicola* NRRL 13618, *F. bactridioides* NRRL 20476, *F. verticillioides* NRRL 20956, *F. subglutinans* NRRL 22016, *F. thapsinum* NRRL 22045, *F. proliferatum* NRRL 22944, *F. sterilihyphosum* NRRL 25623, *F. commune* NRRL 28387 and *F. oxysporum* NRRL 34936. DNA for these species was obtained from the USDA Agricultural Research Service Culture Collection. PCRs were also performed on slash pine DNA in triplicate using the same PCR conditions as the seed screening PCR. A non-template negative control and a positive control of *F. circinatum* DNA were also included.

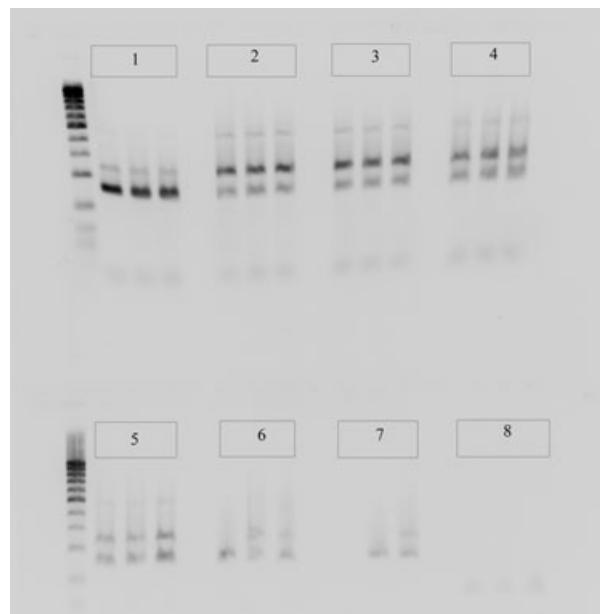


Fig. 1. Real-time PCR amplicons, in triplicate, visualized on a 1.5% (g/ml) agarose gel showing multiple longer than expected amplicons, target size of 146 bp, using primers FCIR-F, FCIR-R and probe FCIR-P with different amounts of *Fusarium circinatum* DNA ranging from (1) 1 ng, (2) 0.1 ng, (3) 0.01 ng, (4) 0.001 ng, (5) 0.0001 ng, (6) 0.00001 ng, (7) 0.000001 ng to (8) No DNA negative control. The ladder on the left has bands from 25 to 1000 bp with a dark band at 500 bp.

2.6 Blotter paper seed screening method

The protocol for the blotter paper seed screening method can be found in the International Seed Testing Association Seed Health Testing Methods (Don 2002) and was originally developed by Anderson (1986). A summary of the method is presented here. Blue blotter paper (House of Doolittle, Chicago, IL, USA) was cut to fit inside 125 × 125 mm autoclavable plastic boxes with transparent lids. The paper, boxes and semi-selective PCNB (pentachloronitrobenzene) broth medium (Don 2002) were autoclaved and then transferred to a laminar flow hood where all culture work was conducted. One piece of blotter paper was placed inside each box and 12 ml of PCNB medium evenly applied to the paper. After the medium was absorbed by the paper, 25 seeds (400 seeds per seed lot total) were placed on the paper and cracked/crushed using a flame-sterilized aluminium template seed crusher. Two drops of PCNB medium were applied to each of the cracked seeds to ensure that seed contents were treated to prevent or slow the growth of most contaminating fungi and bacteria. The lids were sealed with Parafilm[®], and the boxes were placed in a growth chamber set at 20°C with a 12-h photoperiod for 7–10 days. All suspect *Fusarium* colonies were transferred to carnation water agar (CWA) [20 ml of 2- to 4-mm carnation leaf fragments and 15 g agar in 1 l of H₂O (Fisher et al. 1982)] and incubated at 20°C with a 12-h photoperiod for 7–10 days before colonies were identified morphologically as described by ISTA (Don 2002) and Anderson (1986). The ISTA guidelines do not require/recommend subculturing suspect *F. circinatum* colonies on CWA, but this step is included in operational screening at the RSC (J. Bronson, personal communication) and was carried out in this study to promote the development of morphological features important for differentiation of *Fusarium* species and to help differentiate between the large number of intermingled fungal colonies that rapidly grew out of the seed.

2.7 Pathogenicity testing of isolates

Slash pine seedlings from the half-sib family FA2 were grown from seed in a greenhouse for 6 months without the use of pesticides. Twelve of the morphologically identified *F. circinatum* isolates from the blotter paper seed screening method were arbitrarily selected for inclusion in the pathogenicity test using suspect *F. circinatum* isolates from each seed lot. The isolates were plated on CWA and grown at 20°C with a 12-h photoperiod for 10 days before the conidia were harvested by washing the plates with 2 ml of sterile distilled water. The inoculations were performed the same day that the conidia were harvested. Suspensions containing both micro- and macroconidia were diluted to 1×10^6 spores per ml in sterile water using a haemocytometer. The pathogenicity experiment was set up as a complete block design with all 12 isolates and a water control present once in each of the six blocks (13 shoots per block on 74 seedlings, four of which were forked and inoculated twice). The shoot and upper portion of each seedling used were surface-sterilized by spraying with 70% ethanol and allowed to dry. The tips of the shoots to be inoculated were excised using sharp flame-sterilized scissors, and within a few seconds, 25 μ l of inoculum (or water control) was placed on the excised shoot tip so that the droplet did not fall off. After the seedling inoculations were completed, aliquots of the inoculum were streaked on PCNB agar plates (Don 2002) (with 15 g agar added before autoclaving) and viewed 18 h later to determine the spore germination rate for each isolate. Inoculated seedlings were maintained in a greenhouse without the use of pesticides. After 3 months' incubation, necrotic lesion lengths were measured. Shoots were then removed and isolation attempted on PCNB agar from flame-sterilized segments of the leading edge of the lesion. Isolation plates were incubated at 20°C with a 12-h photoperiod. After colonies were grown to 1–2 cm in diameter, suspect *F. circinatum* colonies were transferred to CWA for morphological identification as described above.

2.8 Data analysis

Realplex 2.0 software (Eppendorf Inc., Hauppauge, NY, USA) was used to determine the Ct (count threshold) values for each reaction, the melt-curve analysis was used to confirm whether the correct product was amplified, and the regression analysis was used to determine the amount of target DNA in each reaction using a standard curve. PASW Statistics 18 (SPSS Inc., Chicago, IL, USA) was used to perform a Waller–Duncan multiple range test comparing the necrotic lesion length means in the *F. circinatum* pathogenicity study.

3 Results

3.1 Real-time PCR

The CIRC1L/CIRC4L primers produced positive results only in the presence of *F. circinatum* and *F. subglutinans*. For all other DNA samples, the Ct values fell below the limit of detection with some producing multiple sized amplicons also. The CIRC1L/CIRC4L primer combination showed a linear association between Ct and initial DNA concentration (Fig. 2), down to the limit of detection at 1×10^{-5} ng per. *F. circinatum* template DNA was tested from 1 to 1×10^{-6} ng in the real-time PCR; however, at 1×10^{-6} ng, only two of the three replicates amplified. *F. circinatum* DNA was detected in Seed lots 1, 2, 3, 4 and 6. In Seed lot 4, two of the three 400 seed replicates were positive (Table 2). In Seed lot 5, one of the 400 seed replicates amplified but the Ct value was below the limit of detection.

3.2 Blotter paper

Numerous colonies of *Fusarium* spp. and other fungi grew out of seeds on the blotter paper. Suspect *F. circinatum* colonies (owing to the lack of species-level identification) were detected using this method in all seed lots except Seed lot 5 (Table 2, 3).

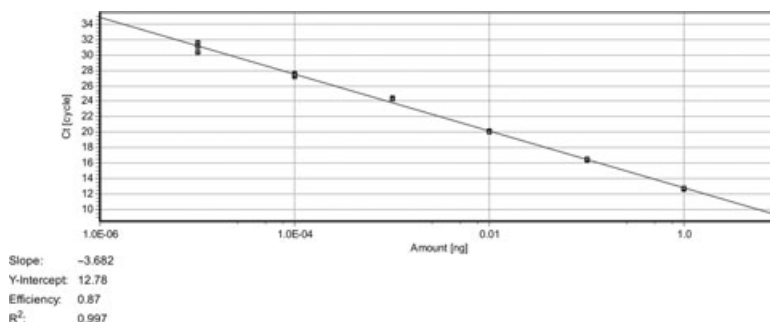


Fig. 2. Standard curve and correlation coefficients for *Fusarium circinatum* DNA, final amounts ranging from 1 to 1×10^{-5} ng per real-time PCR using primers CIRC1L and CIRC4L.

Table 2. Slash pine seed screening for the presence of *F. circinatum* results, using the traditional blotter paper method and new real-time PCR primers CIRC1L and CIRC4L (number of positive samples/number of samples).

Seed lot number	Blotter method	CIRC1L-CIRC4L
1	+	3/3
2	+	3/3
3	+	3/3
4	+ ¹	1/3
5	-	- ²
6	+	3/3

¹One suspect *F. circinatum* isolate was recovered; however, it was found not to be pathogenic after inoculating seedlings.
²One of the three 400 seed replicates was positive for *F. circinatum* but it fell outside of the reliable detection limit of 1×10^{-5} ng.

Table 3. Number of suspect *F. circinatum* isolates retrieved in each seed lot using blotter paper method and the results of the slash pine seedling pathogenicity test indicating the number of *F. circinatum* isolates confirmed.

Seed lot	Number of colonies	Number of isolates pathogenicity tested	Number of pathogenic isolates
1	15	3	3
2	20	3	3
3	7	3	1
4	1	1	0
5	0	0	0
6	2	2	2

Only one suspect *F. circinatum* isolate was found in Seed lot 4 and after inoculating slash pine seedlings, it was found to not be pathogenic which provides evidence, although not conclusive, that the isolate is not *F. circinatum*.

3.3 Pathogenicity testing of isolates

Germination rates of the conidial suspensions were all higher than 83% with an average of 93%. Nine of the 12 isolates, or 75%, used in the inoculations produced a mean necrotic lesion length of 34 mm (standard error = 6.4) and produced typical pitch canker symptoms. These isolates were considered pathogenic to the slash pine and considered to be *F. circinatum*. Three of the 12 isolates, or 25%, used in the inoculation did not produce a significantly greater amount of shoot necrosis length (mean 4 mm, standard deviation of error = 3.0) than the water controls, (Fig. 3) using Waller–Duncan test with Type 1/Type 2 error seriousness ratio = 100, approximately equal to $\alpha = 0.05$. These three isolates were considered to be non-pathogenic and may not be *F. circinatum*. Suspect *F. circinatum* isolates morphologically identified using the ISTA guidelines were re-isolated from 89% of the inoculated shoots.

4 Discussion

Although real-time PCR has been successfully used to detect *F. circinatum* in seeds previously (Ioos et al. 2009), no attempt has been made to apply this methodology to the standard of seed testing required by the ISTA. Our attempts to repeat or successfully modify the published methodology of Ioos et al. (2009) were repeatedly unsuccessful. Thus, we modified the protocol of Schweigkofler et al. (2004). This modification resulted in consistent and highly sensitive detection of *F. circinatum* in or on *P. elliotii* seed – down to at least 1×10^{-5} ng per reaction.

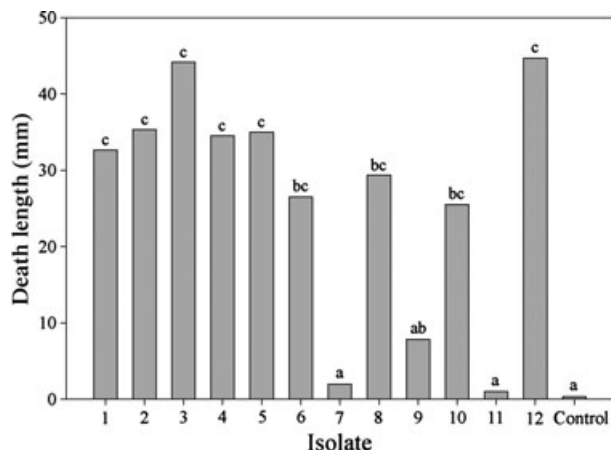


Fig. 3. Lesion length on slash pine seedlings at 12 weeks following inoculation with suspect *F. circinatum* isolates recovered from the blotter paper method. Means separation by Waller-Duncan test with error seriousness ratio = 100.

Table 4. Cost of consumables and the time associated with each seed lot screening method.

Method	Cost per seed lot (\$)	Average elapsed time per seed lot (days)	Man-hours Per Seed lot (hours)
SYBR	3.4	2	2.7
Dual-Labelled Probe	2.3	2	2.7
Blotter Paper	16.6	18	9
Blotter Paper – no subculturing	3.8	10	8.7

Because the goal of this study was to evaluate real-time PCR in the context of the ISTA screening standards, we did not seek to compare *F. circinatum* contamination rates in large numbers of seed lots. Rather, six seed lots were utilized to mirror the maximum number that can be evaluated at one time using the ISTA method as applied at the USDA-APHIS certification of seed lots for international trade (J. Bronson, personal communication).

The value of the real-time PCR-based method is illustrated by tests with Seed lot 4. This method allowed the detection of *F. circinatum* in two of the three 400 seed replicates from Seed lot 4. The blotter paper method may have yielded a false positive for Seed lot 4 because the one suspect *F. circinatum* isolate was found not to be pathogenic, which provides evidence that the isolate is not *F. circinatum*. The blotter paper method may have resulted in the detection of *F. circinatum* from Seed lot 4 if three sets of 400 seeds were screened instead of one, as was the case with the real-time PCR method, but this would add considerable time and cost to the seed screening and is not required by the current ISTA protocol (Don 2002).

The potential for false-negative results with the ISTA method appears to be high based on our study. This protocol calls for suspect *F. circinatum* colonies to be identified directly from the blotter paper. We found this was difficult to accomplish in practice because of the large number of fungi growing out of the seed in close proximity to each other. This made it difficult to distinguish individual colonies, causing *F. circinatum* to be missed because it can be intermingled with other fungi. This problem is exacerbated if suspect colonies are not subcultured early and identified from the subcultures. Therefore, it was necessary to subculture the suspect colonies on CWA before identification. Once suspect colonies are transferred, reliable identification of *F. circinatum* is further complicated by the inconsistent production of coiled hyphae – a distinguishing characteristic of this species (Nirenberg and O'Donnell 1998). The potential for international movement of falsely certified seed lots threatening nurseries, forestry operations and potentially native ecosystems may be increased by reliance on the ISTA methodology. Real-time PCR provides a means to minimize these pitfalls as it is highly sensitive and the method is not affected by the presence of other fungi. Utility of this test could be limited owing to potential false-positive results if *F. subglutinans* is present. However, pines have not been reported as a host of *F. subglutinans* (Leslie and Summerell 2006), implying its presence in pine seed would not be expected.

The potential for false positives using the ISTA methodology is high due to the fact that confirmation of the identity of suspect colonies as *F. circinatum* is not recommended by the protocol. We chose to test the pathogenicity of a subset of the isolates as an additional step to add evidence for the accuracy of our morphological identifications. Indeed, some samples that were tentatively considered positive based on the ISTA protocol were not pathogenic in our test and would therefore not likely be *F. circinatum* based on this criterion.

The cost of consumables and time associated with each seed screening method are given in Table 4. Time can vary widely based on seed contamination levels, facilities and personnel conducting the screening. The blotter paper screening will require a total elapsed time of between 10 and 18 days and take approximately 9 man-hours with a cost of \$4–17 per seed lot, depending on whether suspect colonies are subcultured before identification, whereas the real-time PCR-based method will

take 2 days and 3 man-hours with a cost of \$3 per seed lot. The real-time PCR seed screening method does require expensive and specialized equipment not available in some plant pathology laboratories. The real-time PCR-based method allows more seeds to be screened with less cost and time than the blotter paper method; all of these factors are important in detecting the pathogen.

The detection of *F. circinatum* in two of the three 400 seed replicates in Seed lot 4 using the real-time PCR-based method reveals the importance of which 400 seeds are sampled from a seed lot. If the ISTA guidelines were followed and only 400 seeds were sampled, *F. circinatum* might have been missed in Seed lot 4 resulting in a false negative. This raises the question: How many seeds per seed lot need to be screened? Ioos et al. (2009) stated that there is a need for a statistically sound sampling method for detecting *F. circinatum* that might be unevenly distributed within a seed lot. The results from Seed lot 4 reinforce this need.

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