A Set of Conventional and Multiplex Real-Time PCR Assays for Direct Detection of *Elsinoë fawcettii*, *E. australis*, and *Pseudocercospora angolensis* in Citrus Fruits

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Abstract

Elsinoë fawcettii, *E. australis*, and *Pseudocercospora angolensis* are causal agents of citrus scab and spot diseases. The three pathogens are listed as quarantine pests in many countries and are subject to phytosanitary measures to prevent their entry. Diagnosis of these diseases based on visual symptoms is problematic, as they could be confused with other citrus diseases. Isolation of *E. fawcettii*, *E. australis*, and *P. angolensis* from infected tissues is challenging because they grow slowly on culture media. This study developed rapid and specific detection tools for the in planta detection of these pathogens, using either conventional PCR or one-tube multiplex real-time PCR. Primers and hybridization probes were designed to target the single-copy protein-coding gene *MS204* for *E. fawcettii* and *E. australis* and the translation elongation factor (*Tef-1α*) gene for *P. angolensis*. The specificity of the assays was evaluated by testing against DNA extracted from a large number of isolates (102) collected from different citrus-growing areas in the world and from other hosts. The newly described species *E. citricola* was not included in the

specificity test due to its unavailability from the CBS collection. The detection limits of conventional PCR for the three pathogens were 100, 100, and 10 pg μL^{-1} gDNA per reaction for *E. fawcettii*, *E. australis*, and *P. angolensis*, respectively. The quadruplex qPCR was fully validated assessing the following performance criteria: sensitivity, specificity, repeatability, reproducibility, and robustness. The quadruplex real-time PCR proved to be highly sensitive, detecting as low as 243, 241, and 242 plasmidic copies (pc) μL^{-1} of *E. fawcettii*, *E. australis*, and *P. angolensis*, respectively. Sensitivity and specificity of this quadruplex assay were further confirmed using 176 naturally infected citrus samples collected from Ethiopia, Cameroon, the United States, and Australia. The quadruplex assay developed in this study is robust, cost-effective, and capable of high-throughput detection of the three targets directly from citrus samples. This new detection tool will substantially reduce the turnaround time for reliable species identification and allow rapid response and appropriate action.

Scab diseases of citrus and *Pseudocercospora* leaf and fruit spot are serious diseases of major phytosanitary and economic importance to the citrus industry globally. These diseases considerably reduce the fruit quality and the market value, resulting in significant economic losses in many citrus-production areas (Tan et al. 1996; Yesuf 2013). Scab diseases of citrus are caused mainly by two fungal species: *Elsinoë fawcettii* (syn. *Sphaceloma fawcettii*) is the causal agent of citrus scab (syn. sour orange scab or common scab), and *E. australis* (syn. *S. australis*) causes sweet orange scab disease (SOS) (Whiteside 1978). In each species, different pathotypes have been described based on their host specificity among citrus species (Hou et al. 2014; Hyun et al. 2009; Miles et al. 2015). *E. fawcettii* is cosmopolitan in many humid citrus cultivation areas and affects fruit, leaves, and twigs of many citrus species (Timmer et al. 1996). Unlike *E. fawcettii*, *E. australis* is mainly restricted to southern South America and affects the fruit but not the leaves of most sweet oranges and mandarins (Timmer et al. 1996).

Additionally, *Pseudocercospora* leaf and fruit spot (PLFS), caused by *Pseudocercospora angolensis* (Pretorius et al. 2003), has become more problematic for citrus production. The pathogen is one of the

most significant threats to all citrus species in Africa. The disease was first reported in Angola and Mozambique in 1952 (De Cavalho and Mendes 1953). Since then, it has rapidly spread northward and eastward to 22 countries in Africa (Yesuf 2013), and outside the African mainland to the Comoros in the Indian Ocean and to Yemen in the Arabian Peninsula. However, the disease has not yet been reported in the major citrus producing countries in Africa and represents a potential threat to Egypt, Algeria, Morocco, and South Africa, especially in the warm humid areas.

E. fawcettii, *E. australis*, and *P. angolensis* are not present in Euro-Mediterranean citrus-producing countries. The three pathogens are listed as quarantine pathogens for many countries; therefore, they are subject to strict phytosanitary controls (EFSA PLH Panel 2017a, b). The risk posed by these fungi is that they could be introduced by infected propagative materials and fruit (EFSA PLH Panel 2017a, b). Management of these diseases can be challenging and expensive, and countries where the diseases are not present should focus on prevention through stringent quarantine measures. To facilitate quarantine decisions, it is important to have rapid, specific, and sensitive detection methods for regulatory purposes that will help to prevent introduction of these pathogens into new areas.

Diagnosis of scab diseases based on symptoms is sometimes ambiguous for nonspecialists and the diseases could be easily confused with each other and also with other diseases like Asiatic citrus canker (caused by *Xanthomonas citri* subsp. *citri*) or with those caused by other nonregulated pathogens such as *Diaporthe citri* (causal agent of melanose) (EFSA PLH Panel 2017a) and by various mechanical injuries (Hou et al. 2013). Moreover, isolation of *E. fawcettii*, *E. australis*, and *P. angolensis* from infected tissues is difficult. Fungi of the genus *Elsinoë* are notoriously difficult to isolate from tissue that is not fresh, such as fruit shipped for export, and isolations from such samples are often inconclusive (Fan et al. 2017). Like the scab fungi, *P. angolensis* grows slowly on culture media, and colony

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development may require 21 days of incubation (Brentu et al. 2013) and is often outgrown by other fast-growing fungi inhabiting the same citrus tissues. Consequently, false negative results can be frequent with this method. *E. fawcettii* and *E. australis* cannot be differentiated based on morphological features (Timmer et al. 1996), while they could be clearly distinguished by host range and molecular analysis (Hyun et al. 2009). The two species can be successfully differentiated by restriction analysis of internal transcribed spacer (ITS) rDNA, random amplified polymorphic DNA (RAPD), and by sequence analysis of ITS, Translation elongation factor 1- α (*Tef1*- α), and 28S nrDNA (LSU) (Fan et al. 2017; Hou et al. 2014; Hyun et al. 2009). For *P. angolensis*, the nucleotide sequence data of ITS, β -tubulin, LSU, Actin (*Act*), Calmodulin (*Cal*), *Tef1*- α , and RNA polymerase II second largest subunit (*rpb2*) have demonstrated their usefulness as markers for differentiating *P. angolensis* from other *Pseudocercospora* taxa (Crous et al. 2013; Quaadvlieg et al. 2012).

Few molecular methods are currently available for the direct detection of these pathogens in host plant tissues. Hyun et al. (2007) developed a conventional PCR test for detecting several pathotypes of *E. fawcettii* and *E. australis* in culture media and in citrus tissues; however, to our knowledge no specific test targeting *P. angolensis* has been described. Real-time PCR (qPCR) now offers the possibility to develop more rapid, specific, and sensitive assays for diagnostic purposes, and has been used successfully to detect many fungal quarantine pathogens (Aguayo et al. 2017; Boutigny et al. 2013; Guinet et al. 2015; Ioos et al. 2012), including *E. fawcettii* (Hou et al. 2013). However, data are lacking regarding the specificity of the latter test, since no *E. australis* strain was included for the assay validation.

DNA sequences of nuclear ribosomal rDNA (18S, 28S, ITS, IGS), mitochondrial DNA (Cytochrome Oxidase I and II), and protein-coding genes (β -tubulin, RNA polymerases, γ -actin) are commonly used in phylogenetic studies and to design PCR primers for detection purposes (Schmitt et al. 2009). Loci with high copy numbers are suitable for PCR amplification and reliable detection. However, they have the potential of being phylogenetically misleading (Schmitt et al. 2009). For instance, nonorthologous copies of ITS region between strains within the same species have been described (Ko and Jung 2002). In addition, the number of copies might fluctuate with the pathogen growth stage (Atallah et al. 2007). Many single-copy protein-coding genes were defined for recovering robust phylogenies and resolving species relationships (Aguileta et al. 2008; Feau et al. 2011; Schmitt et al. 2009; Walker et al. 2012). Moreover, the usefulness of using single-copy protein-coding genes to develop detection assays for many regulated fungal pathogens has been successfully validated (Boutigny et al. 2013; Ioos et al. 2006; Schenck et al. 2016). In this context, we assumed that those single-copy protein-coding genes are suitable candidates for developing primers and probe specific to *E. fawcettii* and *E. australis* in *sensu lato* regardless of their pathotypes.

The aims of this study were to (i) develop a set of specific, sensitive, and robust PCR assays to identify and detect *E. fawcettii*, *E. australis* (*sensu lato*), and *P. angolensis* using either conventional or real-time PCR approaches, (ii) evaluate the performance criteria of qPCR assays including repeatability, reproducibility, transferability, and robustness, and (iii) validate the efficiency of these new tools on a large set of naturally infected citrus samples.

Materials and Methods

Fungal isolates and DNA extraction. The subset of isolates used for the screening of the tentative PCR primers and probe included 32 isolates of *E. fawcettii*, six isolates of *E. australis*, six isolates of *P. angolensis*, taxa phylogenetically close to the target species, as well as other genera commonly encountered on citrus fruit (Table 1). The identity of each isolate was confirmed by PCR amplification and sequence analysis of the internal transcribed spacer (ITS) region of rDNA using the ITS1/ITS4 primers (White et al. 1990). All the isolates were handled in level 3 biohazard containment facilities at

mycology unit-ANSES. Total DNA was extracted from fungal cultures using a commercial plant DNA extraction kit (DNeasy Plant Mini Kit, Qiagen, Germany). The isolates were grown on potato dextrose agar (PDA) at 27°C for 1 to 2 weeks depending on the growth rate of each fungus. Then the aerial mycelium was scraped from the culture surface using a sterile scalpel blade and transferred into 2-ml Lysing Matrix A microtubes containing a silica sphere and a garnet matrix (MP Biomedicals, Santa Ana, CA), with 400 μ l of AP1 lysis buffer provided by the DNA extraction kit. The mycelium was homogenized at 6.5 Hz for 30 s using the FastPrep 24 instrument (MP Biomedicals). Total genomic DNA (gDNA) was extracted following the manufacturer's protocol. Purified fungal DNA was measured by the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) then adjusted to 1 ng μ l⁻¹ with AE elution buffer and stored at -30°C until further analysis.

Design of specific primers and hydrolysis probes. For *E. fawcettii* and *E. australis*, the partial sequences of the ITS region of rDNA (207 sequences for 14 *Elsinoë* spp.), translation elongation factor 1 α (*Tef1*- α) gene (97 sequences for five *Elsinoë* spp.), 28S large subunit ribosomal RNA gene (eight sequences for 4 *Elsinoë* spp.), and β -tubulin (two sequences for *E. fawcettii*) loci were retrieved from the National Center for Biotechnology Information (NCBI) database. For each locus, the sequences were evaluated to pinpoint regions with high polymorphism between the two species. All the above-mentioned loci showed no suitable regions to design primers and probes due to thermodynamic and polymorphism constraints. There was either a low level of interspecific variability between *E. fawcettii* and *E. australis* or a high level of intraspecific polymorphism among the different pathotypes of the same species. Therefore, sequences of four single-copy protein-coding orthologous genes (*Tsr1*, *Mcm7*, *MS204*, and *FG740*) were generated by PCR for nine isolates of *Elsinoë* species. PCR amplification of the genes was carried out according to Schmitt et al. (2009) for the *Tsr1* and *Mcm7* genes, Walker et al. (2012) for the *MS204* gene, and Husson et al. (2011) for the *FG740* gene. Sequences from each of the four markers were analyzed and aligned by MUSCLE (Edgar 2004) in the Geneious software R6 (Biomatters, New Zealand) and manually examined to distinguish suitable interspecific polymorphic regions. The interspecific polymorphisms observed within the *MS204* gene sequences suggested that this marker was a good candidate for designing specific primers and probes. The sequences of the *MS204* gene generated during this study were deposited in GenBank (Table 2). A series of tentative primers and hydrolysis probe combinations specific to *E. fawcettii* and *E. australis* were designed from polymorphic regions of the *MS204* gene (Table 3) using PRIMER 3 in the Geneious software R6. To avoid nonspecific cross hybridizations of the oligonucleotides under multiplex conditions, primers and probes specific to *E. fawcettii* and *E. australis* were derived from two different regions of the *MS204* gene sequence. The melting temperature, potential formation of secondary structures, and interactions among the oligonucleotide sequences were evaluated *in silico* using the same software and PriDimerCheking software (Shen et al. 2010). BLASTN analysis on NCBI database was performed to evaluate the primers and probe specificity against other closely related genome sequences. For *P. angolensis*, specific primers and probe (Table 3) were designed as described above based on sequence of Translation elongation factor (*Tef1*- α). This gene has been successfully used to distinguish taxonomic species within *Pseudocercospora* and in phylogenetic studies (Crous et al. 2013; Quaadvlieg et al. 2012; Silva et al. 2016). All primers and probes used in this study were synthesized by Eurogentec (Seraing, Belgium). A preliminary *in vitro* evaluation of specificity of the candidate primers was performed in conventional PCR using small subset of 1 ng μ l⁻¹ gDNA of target and nontarget species including *E. fawcettii* (CBS 233.64, CBS 232.64), *E. australis* (CBS 314.32, CBS 313.32, CBS 229.64, LSVM 631), *E. veneta* (CBS 150.27), *E. eucaalypticola* (CBS 124765), *E. centrolobi* (CBS 222.50), *P. angolensis* (CBS 112748, MC -39, C-ethiop-3), *P. fijiensis* (LSVM 501), *P. musicola* (LSVM 511), and *P. eumusae* (LSVM 536).

Conventional PCR amplification. The species-specific conventional PCR assays were performed in a 20- μ l reaction volume containing 1 $\times$ PCR reaction buffer (HGS diamond Taq, Eurogentec), 1.5 mM MgCl₂, 4 $\times$ 0.25 mM dNTPs, 0.5 μ M each of the forward and reverse primers, 0.05 U of HGS diamond Taq (Eurogentec), 2 μ l of template DNA (1 ng μ l⁻¹), and molecular-grade water to 20 μ l. The PCR reactions were performed in a 96-well plate and carried out on a GenAmp 9700 thermocycler (Applied Biosystems, Foster City, CA). The cycling conditions included an initial denaturation step at 95°C for 10 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s (for *E. fawcettii* and *E. australis* primers) or 54°C for 30 s (for *P. angolensis* primers), and elongation at 72°C for 60 s; and a final extension at 72°C for 7 min. After amplification, 10 μ l of PCR product was separated by electrophoresis on a 1.5% agarose gel in 0.5 $\times$ TBE buffer along with a 100-bp DNA ladder at 100 V for 1 h. Then the gels were stained for 20 min with ethidium bromide and analyzed under UV light.

Implementation of real-time PCR quality controls. The amplicons amplified by PCR using the primer pairs E.faw-F/R, E.aus-F/R, and P.ango-F/R for *E. fawcettii* CBS 233.64, *E. australis* CBS 299.64, and *P. angolensis* CBS 112748 gDNA, respectively, were each inserted into a pCR4-TOPO vector (Invitrogen), which was used to transform chemically competent TOP10 cells (Invitrogen), according to the manufacturer's instructions. The bacterial cells were then subcultured overnight at 37°C and the plasmids were purified using a Nucleospin Plasmid Kit (Macherey-Nagel). Based on the sequences of pCR4-TOPO vector and the amplicon sequences of the three target species, the molecular weight of the individual subcloned plasmid was determined, and the number of target plasmidic copies (pc) per microliter was calculated. A calibrated stock solution and 10-fold serial dilution in 1 $\times$ Tris EDTA buffer were prepared for each plasmidic clone of *E. australis*, *E. fawcettii*, and *P. angolensis*, and stored at -20°C until further use. These plasmidic clones of *E. australis* and *E. fawcettii* could be used as species-specific positive controls in both the PCR and qPCR assay, since the target regions for the real-time forward and reverse primers and for the probe were located within the region bounded by the conventional PCR forward and reverse primers (Table 3).

Monoplex and quadruplex real-time PCR assays. The Rotor-Gene 6500 (Corbett Research, Mortlake, Australia) was used for real-time PCR amplifications. The instrument was set with an auto-gain optimization for each channel performed before the first fluorescence acquisition. The monoplex (i.e., targeting one single pathogen) real-time PCR reactions were performed in a total volume of 20 μ l using the Mastermix No Rox kit (Eurogentec, Seraing, Belgium) and the mixture contained 1 $\times$ reaction buffer, 0.3 μ M of each forward and reverse primer, 0.1 μ M of probe, 2 μ l of template DNA (1 ng μ l⁻¹) and molecular-grade water to 20 μ l. Real-time PCR was performed under the following conditions: an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, and annealing/elongation at 60°C for 60 s. In each reaction, the cycle threshold (Ct) value was determined using the Rotor-Gene software version 1.7.75, and the threshold line was fixed manually at 0.02. Each DNA sample was tested in duplicate and the mean and standard deviation for the Ct values were computed. The multiplex assay consisted of a real-time PCR reaction in which DNA template from the three different pathogens could be detected in a single tube/reaction. In the quadruplex real-time assays, the master mix contained equal concentrations of each of *E. fawcettii*, *E. australis*, and *P. angolensis*, forward, reverse primers, and probes in addition to the 18S Uni primers and probe combination (Table 3) that targets conserved regions of the 18S rDNA of plants and fungi, and serves as a quality control for the DNA extraction (Ioos et al. 2009). Preliminary experiments were carried out to confirm the possibility to multiplex the four primers and probes without compromising test's sensitivity and PCR efficiency (*data not shown*). The master mixture and amplification conditions were as described above with monoplex real-time PCR. For easy manipulation and for reducing the pipetting errors, a bulk ready-to-use mix of quadruplex primers and probes was prepared.

Analytical specificity and inclusivity of conventional and real-time PCR assays. To verify the specificity, each conventional and qPCR assay developed in this study was assessed with 102 DNA extracts from nontarget species, comprising genetically related fungal taxa, and fungal species commonly found on citrus tissue (Table 1). Each assay was also evaluated using several sources of target DNA, from various species of citrus fruit and regions. All DNA extracts were normalized to a common concentration of 1 ng μ l⁻¹ prior to testing. DNA extracted from healthy citrus tissues was used as an external negative control to exclude any cross-reaction of the primers and probes with citrus DNA. The inclusivity of the developed tests was verified with DNA of 32 *E. fawcettii* isolates, six *E. australis* isolates, and six *P. angolensis* isolates.

Assessment of performance criteria. The analytical sensitivity of conventional PCR was assessed with serial dilutions of *E. fawcettii* CBS 233.64, *E. australis* CBS299.64, and *P. angolensis* CBS 112748 genomic DNA ranging from 0.5 ng μ l⁻¹ to 0.5 fg μ l⁻¹. For qPCR assay, the sensitivity was assessed using a 10-fold dilution series of the plasmidic DNA (pDNA) positive controls diluted in 1 $\times$ Tris-EDTA buffer and in two backgrounds of citrus DNA. The citrus DNA extract was obtained from the fruit rinds of sweet orange (*Citrus sinensis*) and lemon (*Citrus limon*) and was adjusted to 1 ng μ l⁻¹. The dilution series ranged from 2.43 $\times$ 10⁷ to 243 pc μ l⁻¹, 2.41 $\times$ 10⁷ to 241 pc μ l⁻¹, and 2.42 $\times$ 10⁷ to 242 pc μ l⁻¹ for *E. fawcettii*, *E. australis*, and *P. angolensis*, respectively. Each target was tested alone in TE matrix and mixed with the other two target species in TE and citrus matrices. The limit of detection (LOD) was determined as the minimal quantity of target DNA that could be consistently amplified by PCR or qPCR. For each qPCR assay, a standard curve was constructed and the corresponding amplification efficiency was computed.

Repeatability of the qPCR assay was evaluated with 10 replicates of different pDNA concentrations set at 10 $\times$ LOD, 100 $\times$ LOD, and 1000 $\times$ LOD, as well as a comparison of 0.1 ng μ l⁻¹ fungal gDNA all diluted in a background of 1 ng μ l⁻¹ of citrus fruit gDNA. Reproducibility was tested with one replicate of the same DNA concentration during an individual run, and additionally in 10 different runs over 2 weeks by three different operators and two Rotor-Gene thermal cyclers. For each combination, intra- and interassay coefficient of variation (CV) was computed.

Since the multiplex qPCR assay was initially developed with the Rotor-Gene platform, the transferability to other equipment, such as the LightCycler 480 (Roche), needed to be tested. The qPCR transferability was assessed by comparing 10 replicates of different target pDNA concentrations set at 10 $\times$ LOD, 100 $\times$ LOD, and 10 replicates of 0.1 ng μ l⁻¹ gDNA of *E. fawcettii* CBS 233.64, *E. australis* CBS 299.64, *P. angolensis* CBS 112748, and *P. fijiensis* (LSVM 501) as the DNA specificity control. The PCR conditions were the same with the LightCycler as for the Rotor-Gene described above.

To examine the robustness of the qPCR assay, several reaction parameters were deliberately modified and the assay was carried out using 10 replicates of different pDNA concentrations close to the limit of detection, i.e., 10 $\times$ LOD and 100 $\times$ LOD, as well as with 10 replicates of 0.1 ng μ l⁻¹ target species gDNA diluted in a background of 1 ng μ l⁻¹ of citrus fruit DNA. The robustness of qPCR assay was challenged with a $\pm$ 10% variation of the reaction volume or DNA template volume, and slight variations of the hybridization temperature ($\pm$ 2°C).

Analysis of naturally infected citrus materials. Citrus samples displaying scab or PLFS symptoms collected on different continents were evaluated by (i) in planta direct detection of the three pathogens using the molecular assays developed in this study, and when feasible (ii) plating on culture medium followed by the assays from this study of the fungal colonies. Total DNA was extracted from different types of citrus species and organs. Individual lesions on fruit skin or on leaves (3- to 4-mm-dia. portions) were collected using a sterile scalpel blade and cut in halves when the lesion was large enough; otherwise, the entire lesion was taken for direct molecular testing. The first halves were transferred to a Lysing

Table 1. List of fungal, oomycete, and bacterial species used in this study^a

Species	Isolate	Host	Origin	Year	Conventional PCR ^b			Multiplex qPCR (mean Ct value $\pm$ SD)			ITS GenBank accession number
					<i>Ef</i> *	<i>Ea</i> **	<i>Pa</i> ***	<i>Ef</i>	<i>Ea</i>	<i>Pa</i>	
<i>Elsinoë fawcettii</i>	CBS 232.64	<i>Citrus limonium</i>	Florida	1964	Pos	Neg	Neg	21.21 $\pm$ 0.09	-	-	MG956727
<i>E. fawcettii</i>	CBS 233.64	<i>Citrus aurantium</i>	Panama	1964	Pos	Neg	Neg	17.96 $\pm$ 0.10	-	-	MG956728
<i>E. fawcettii</i>	Efaw-MUR4	<i>Citrus reticulata</i>	Florida	2001	Pos	Neg	Neg	18.37 $\pm$ 0.04	-	-	MG956729
<i>E. fawcettii</i>	PK-MUR-L 1	<i>Citrus reticulata</i>	Florida	2014	Pos	Neg	Neg	19.69 $\pm$ 0.24	-	-	MG956730
<i>E. fawcettii</i>	USA-Russel-15	<i>Citrus reticulata</i> $\times$ <i>sinensis</i> Temple	Florida	1991	Pos	Neg	Neg	17.89 $\pm$ 0.00	-	-	MG956731
<i>E. fawcettii</i>	36954	<i>Citrus reticulata</i> $\times$ <i>sinensis</i> Temple	Florida	1988	Pos	Neg	Neg	18.42 $\pm$ 0.00	-	-	MG956732
<i>E. fawcettii</i>	Briggs-3 Benlate	<i>Citrus paradisi</i>	Florida	2003	Pos	Neg	Neg	19.36 $\pm$ 0.04	-	-	MG956733
<i>E. fawcettii</i>	CAL-WH-2	<i>Citrus microcarpa</i> Bunge	Florida	2002	Pos	Neg	Neg	19.03 $\pm$ 0.13	-	-	MG956734
<i>E. fawcettii</i>	CARRIZO	<i>Carrizo citrange</i>	Florida	1996	Pos	Neg	Neg	19.14 $\pm$ 0.04	-	-	MG956735
<i>E. fawcettii</i>	CC-1	<i>Citrus paradisi</i>	Florida	1990	Pos	Neg	Neg	19.21 $\pm$ 0.00	-	-	MG956736
<i>E. fawcettii</i>	CC-3	<i>Citrus volkameriana</i>	Florida	1990	Pos	Neg	Neg	26.07 $\pm$ 0.35	-	-	MG956737
<i>E. fawcettii</i>	Citro-1	<i>Citrus microcarpa</i> Bunge	Florida	1993	Pos	Neg	Neg	22.26 $\pm$ 0.13	-	-	MG956738
<i>E. fawcettii</i>	CON-LAL-2	<i>Citrus microcarpa</i> Bunge	Florida	2001	Pos	Neg	Neg	26.33 $\pm$ 0.23	-	-	MG956739
<i>E. fawcettii</i>	DUDA-IMK-TEM-1	<i>Citrus reticulata</i> $\times$ <i>sinensis</i> Temple	Florida	2001	Pos	Neg	Neg	22.27 $\pm$ 0.08	-	-	MG956740
<i>E. fawcettii</i>	DUDA-IMK-TEM-2	<i>Citrus reticulata</i> $\times$ <i>sinensis</i> Temple	Florida	2001	Pos	Neg	Neg	19.11 $\pm$ 0.01	-	-	MG956741
<i>E. fawcettii</i>	DUDA-IMK-TEM-3	<i>Citrus reticulata</i> $\times$ <i>sinensis</i> Temple	Florida	2001	Pos	Neg	Neg	19.11 $\pm$ 0.01	-	-	MG956742
<i>E. fawcettii</i>	DUDA-IMK-TEM-5	<i>Citrus reticulata</i> $\times$ <i>sinensis</i> Temple	Florida	2001	Pos	Neg	Neg	19.88 $\pm$ 0.01	-	-	MG956743
<i>E. fawcettii</i>	EUS-MUR-2	<i>Citrus reticulata</i>	Florida	2001	Pos	Neg	Neg	19.16 $\pm$ 0.12	-	-	MG956744
<i>E. fawcettii</i>	EUS-MUR-3	<i>Citrus reticulata</i>	Florida	2001	Pos	Neg	Neg	19.18 $\pm$ 0.01	-	-	MG956745
<i>E. fawcettii</i>	FT.Meade-2 FL	<i>Citrus paradisi</i>	Florida	Unknown	Pos	Neg	Neg	18.28 $\pm$ 0.21	-	-	MG956746
<i>E. fawcettii</i>	Fort.Pierce-3a	<i>Citrus reticulata</i> $\times$ <i>sinensis</i> Temple	Florida	Unknown	Pos	Neg	Neg	22.24 $\pm$ 0.01	-	-	MG956747
<i>E. fawcettii</i>	Ft- Station-TEM-4	<i>Citrus reticulata</i> $\times$ <i>sinensis</i> Temple	Florida	2001	Pos	Neg	Neg	19.42 $\pm$ 0.01	-	-	MG956748
<i>E. fawcettii</i>	HMT-RG-40C-1	<i>Citrus paradisi</i>	Florida	2005	Pos	Neg	Neg	19.31 $\pm$ 0.02	-	-	MG956749
<i>E. fawcettii</i>	HMT-RG-40C-4	<i>Citrus paradisi</i>	Florida	2005	Pos	Neg	Neg	20.32 $\pm$ 0.09	-	-	MG956750
<i>E. fawcettii</i>	HMT-RG-40C-7	<i>Citrus paradisi</i>	Florida	2005	Pos	Neg	Neg	19.30 $\pm$ 0.01	-	-	MG956751
<i>E. fawcettii</i>	HMT-RG-40C-5	<i>Citrus paradisi</i>	Florida	2005	Pos	Neg	Neg	19.18 $\pm$ 0.09	-	-	MG956752
<i>E. fawcettii</i>	May 1	<i>Citrus paradisi</i>	Florida	Unknown	Pos	Neg	Neg	19.94 $\pm$ 0.07	-	-	MG956753
<i>E. fawcettii</i>	May 2	<i>Citrus paradisi</i>	Florida	Unknown	Pos	Neg	Neg	19.20 $\pm$ 0.05	-	-	MG956754
<i>E. fawcettii</i>	May 3	<i>Citrus paradisi</i>	Florida	Unknown	Pos	Neg	Neg	20.11 $\pm$ 0.15	-	-	MG956755
<i>E. fawcettii</i>	PK-MUR-L3	<i>Citrus reticulata</i>	Florida	2014	Pos	Neg	Neg	23.22 $\pm$ 0.02	-	-	MG956756
<i>E. fawcettii</i>	RUSELL-5	<i>Citrus reticulata</i> $\times$ <i>sinensis</i> Temple	Florida	Unknown	Pos	Neg	Neg	19.26 $\pm$ 0.03	-	-	
<i>E. fawcettii</i>	RUSELL-15	<i>Citrus reticulata</i> $\times$ <i>sinensis</i> Temple	Florida	1991	Pos	Neg	Neg	20.00 $\pm$ 0.07	-	-	
<i>E. australis</i>	CBS 229.64	<i>Citrus aurantiifolia</i>	Brazil	Unknown	Neg	Pos	Neg	-	24.08 $\pm$ 0.35	-	MG956757
<i>E. australis</i>	CBS 313.32	<i>Citrus aurantium</i>	Brazil	Unknown	Neg	Pos	Neg	-	25.18 $\pm$ 0.10	-	MG956758
<i>E. australis</i>	CBS 314.32	<i>Citrus aurantium</i>	Brazil	Unknown	Neg	Pos	Neg	-	24.44 $\pm$ 0.16	-	MG956759
<i>E. australis</i>	1629	<i>Citrus spp.</i>	Uruguay	2012	Neg	Pos	Neg	-	37.02 $\pm$ 0.33	-	MG956760
<i>E. australis</i>	SOS 44810-1s	<i>Citrus aurantium</i>	Florida	2011	Neg	Neg	Neg	-	31.4 $\pm$ 0.02	-	
<i>E. australis</i>	SOS 53525	<i>Citrus aurantium</i>	Florida	2012	Neg	Neg	Neg	-	30.89 $\pm$ 0.30	-	MG956761
<i>E. veneta</i>	CBS 150.27	<i>Rosa sp.</i>	Unknown	Unknown	Neg	Neg	Neg	-	-	-	
<i>E. centrolobi</i>	CBS 222.50	<i>Centrolobium</i> <i>robustum</i>	Brazil	Unknown	Neg	Neg	Neg	-	-	-	MG956762
<i>E. eucalypticola</i>	CBS 124765	<i>Eucalyptus sp.</i>	Australia	2006	Neg	Neg	Neg	-	-	-	
<i>Pseudocercospora angolensis</i>	CBS 112748	<i>Citrus sp.</i>	Zimbabwe	Unknown	Neg	Neg	Pos	-	-	20.84 $\pm$ 0.01	MG956763
<i>P. angolensis</i>	CBS 112933	<i>Citrus sp.</i>	Zimbabwe	2002	Neg	Neg	Pos	-	-	20.77 $\pm$ 0.34	MG956764
<i>P. angolensis</i>	CBS 149.53	<i>Citrus sinensis</i>	Angola	Unknown	Neg	Neg	Pos	-	-	20.55 $\pm$ 0.09	MG956765
<i>P. angolensis</i>	MC-39	<i>Citrus aurantium</i>	Ghana	2004	Neg	Neg	Pos	-	-	21.91 $\pm$ 0.17	MG956766
<i>P. angolensis</i>	C-ethiop-1	<i>Citrus aurantiifolia</i>	Ethiopia	2015	Neg	Neg	Pos	-	-	21.76 $\pm$ 0.34	MG956767
<i>P. angolensis</i>	C-ethiop-3	<i>Citrus aurantiifolia</i>	Ethiopia	2015	Neg	Neg	Pos	-	-	18.66 $\pm$ 0.00	MG956768
<i>P. fijensis</i>	LSVM 501	<i>Musa sp.</i>	Guadeloupe Island	2012	Neg	Neg	Neg	-	-	-	MG956769
<i>P. musicola</i>	LSVM 511	<i>Musa sp.</i>	Guadeloupe Island	2012	Neg	Neg	Neg	-	-	-	
<i>P. eumusae</i>	487 THA	<i>Musa sp.</i>	Thailand	2012	Neg	Neg	Neg	-	-	-	MG956770
<i>Alternaria alternata</i>	C- Ethio-7	<i>Citrus aurantiifolia</i>	Ethiopia	2015	Neg	Neg	Neg	-	-	-	MG956771
<i>Alternaria alternata</i>	CHUR-DAN-R4-F1-1'S	<i>Citrus reticulata</i>	Florida	2010	Neg	Neg	Neg	-	-	-	
<i>Amicosphaerella africana</i>	17NCC3	<i>Citrus sinensis</i>	Panama	2010	Neg	Neg	Neg	-	-	-	
<i>Amicosphaerella africana</i>	19NCC3	<i>Citrus sinensis</i>	Panama	2010	Neg	Neg	Neg	-	-	-	
<i>Cercospora sp.</i>	LSVM 1100	<i>Citrus paradisi</i>	Mexico	2006	Neg	Neg	Neg	-	-	-	MG956772
<i>Cercospora aurantia</i>	LSVM 1265	<i>Citrus sinensis</i>	Brazil	2015	Neg	Neg	Neg	-	-	-	MG956773

(Continued on next page)

^a The isolates were obtained from the culture collections of Mycology Unit-ANSES (France); Citrus Research and Education Center (Florida); CBS-KNAW culture collection (The Netherlands); CIRAD (France) and IVIA (Spain).

^b **E. fawcettii*-specific test. ***E. australis*-specific test. ****P. angolensis*-specific test.

Table 1. (Continued from previous page)

Species	Isolate	Host	Origin	Year	Conventional PCR ^b			Multiplex qPCR (mean Ct value $\pm$ SD)			ITS GenBank accession number
					Ef*	Ea**	Pa***	Ef	Ea	Pa	
<i>Cladosporium</i> sp.	C- Ethiop-4	<i>Citrus aurantiifolia</i>	Ethiopia	2015	Neg	Neg	Neg	-	-	-	MG956774
<i>Cladosporium</i> <i>cladosporioides</i>	LSVM 1175	<i>Citrus grandis</i>	China	2014	Neg	Neg	Neg	-	-	-	MG956775
<i>Colletotrichum</i> <i>gloeosporioides</i>	C- Ethiop-23	<i>Citrus aurantiifolia</i>	Ethiopia	2015	Neg	Neg	Neg	-	-	-	MG956776
<i>Colletotrichum</i> <i>truncatum</i>	C- Ethiop-19	<i>Citrus aurantiifolia</i>	Ethiopia	2015	Neg	Neg	Neg	-	-	-	MG956777
<i>Colletotrichum</i> <i>boninense</i>	LSV M 1162	<i>Citrus sinensis</i>	Argentina	2014	Neg	Neg	Neg	-	-	-	MG956778
<i>Diaporthe</i> sp.	LSVM 1109	<i>Citrus latifolia</i>	Brazil	2012	Neg	Neg	Neg	-	-	-	MG956779
<i>Diaporthe citri</i>	PC-STL-ss	<i>Citrus sinensis</i>	Florida	2001	Neg	Neg	Neg	-	-	-	
<i>Dothistroma</i> <i>septosporium</i>	LSVM 760	<i>Pinus pinaster</i>	France	2013	Neg	Neg	Neg	-	-	-	MG956780
<i>Fusarium</i> <i>kyushuense</i>	C- Ethiop-24	<i>Citrus aurantiifolia</i>	Ethiopia	2015	Neg	Neg	Neg	-	-	-	MG956781
<i>Fusarium</i> <i>oxysporum</i>	C- Ethiop-9	<i>Citrus aurantiifolia</i>	Ethiopia	2015	Neg	Neg	Neg	-	-	-	MG956782
<i>Fusarium</i> <i>proliferatum</i>	LSVM 1168	<i>Citrus sinensis</i>	Argentina	2014	Neg	Neg	Neg	-	-	-	
<i>Lecanosticta</i> <i>acicola</i>	LSVM 257	<i>Pinus radiata</i>	France	1995	Neg	Neg	Neg	-	-	-	MG956783
<i>Neofusicoccum</i> <i>parvum</i>	LSVM 884	<i>Citrus sinensis</i>	Guadeloupe Island	2013	Neg	Neg	Neg	-	-	-	MG956784
<i>Nigrospora</i> <i>sphaerica</i>	LSVM 1144	<i>Citrus latifolia</i>	Guadeloupe Island	2014	Neg	Neg	Neg	-	-	-	MG956785
<i>Nigrospora</i> <i>oryzae</i>	LSVM 1158	<i>Citrus latifolia</i>	Guadeloupe Island	2014	Neg	Neg	Neg	-	-	-	MG956786
<i>Passalora</i> <i>loranthi</i>	LSVM 1114	<i>Citrus sinensis</i>	Brazil	2014	Neg	Neg	Neg	-	-	-	MG956787
<i>Periconia</i> sp.	LSVM 1139	<i>Citrus sinensis</i>	Argentina	2014	Neg	Neg	Neg	-	-	-	MG956788
<i>Penicillium</i> <i>italicum</i>	Penicillium 1	<i>Citrus</i> sp.	Unknown	2016	Neg	Neg	Neg	-	-	-	MG956789
<i>Pestalotiopsis</i> sp.	C- Ethiop-25	<i>Citrus aurantiifolia</i>	Ethiopia	2015	Neg	Neg	Neg	-	-	-	MG956790
<i>Phyllosticta</i> <i>citribraziliensis</i>	LSVM 1098	<i>Citrus</i> sp.	Brazil	2014	Neg	Neg	Neg	-	-	-	MG956791
<i>Phyllosticta</i> <i>capitalensis</i>	LSVM 1117	<i>Citrus latifolia</i>	Guadeloupe Island	2014	Neg	Neg	Neg	-	-	-	MG956792
<i>Phyllosticta</i> <i>capitalensis</i>	GM-KF-F5	<i>Citrus sinensis</i>	Florida	2013	Neg	Neg	Neg	-	-	-	
<i>Phyllosticta</i> <i>capitalensis</i>	GM-33-3'S	<i>Citrus sinensis</i>	Florida	2010	Neg	Neg	Neg	-	-	-	
<i>Phyllosticta</i> <i>citriasiana</i>	LSVM 1146	<i>Citrus maxima</i>	China	2014	Neg	Neg	Neg	-	-	-	MG956793
<i>Phyllosticta</i> <i>citriasiana</i>	LSVM 1147	<i>Citrus maxima</i>	China	2014	Neg	Neg	Neg	-	-	-	MG956794
<i>Phyllosticta</i> <i>citricarpa</i>	LSVM 494	<i>Citrus</i> sp.	Togo	2011	Neg	Neg	Neg	-	-	-	MG956795
<i>Phyllosticta</i> <i>citricarpa</i>	LSVM 1101	<i>Citrus sinensis</i> $\times$ <i>Poncirus trifoliata</i>	South Africa	2014	Neg	Neg	Neg	-	-	-	MG956796
<i>Phyllosticta</i> <i>citricarpa</i>	CBS 141349	<i>Citrus limon</i>	Italy	2015	Neg	Neg	Neg	-	-	-	
<i>Phyllosticta</i> <i>citricarpa</i>	CBS 141350	<i>Citrus sinensis</i>	Malta	2015	Neg	Neg	Neg	-	-	-	
<i>Phyllosticta</i> <i>citricarpa</i>	CBS 141351	<i>Citrus sinensis</i>	Portugal	2015	Neg	Neg	Neg	-	-	-	
<i>Phyllosticta</i> <i>citrichinaensis</i>	LSVM 1094	<i>Citrus</i> sp.	China	2014	Neg	Neg	Neg	-	-	-	MG956797
<i>Phyllosticta</i> <i>paracitricarpa</i>	CBS 141357	<i>Citrus lemon</i>	Greece	2016	Neg	Neg	Neg	-	-	-	
<i>Phyllosticta</i> sp.	LSVM 1163	<i>Citrus grandis</i>	China	2014	Neg	Neg	Neg	-	-	-	MG956798
<i>Phytophthora</i> <i>palmivora</i>	LSVM 1405	<i>Pittosporum tobira</i>	Italy	2016	Neg	Neg	Neg	-	-	-	MG956799
<i>Phoma</i> sp.	C- Ethiop-11	<i>Citrus aurantiifolia</i>	Ethiopia	2015	Neg	Neg	Neg	-	-	-	MG956800
<i>Phomopsis</i> sp.	C- Ethiop-18	<i>Citrus aurantiifolia</i>	Ethiopia	2015	Neg	Neg	Neg	-	-	-	MG956801
<i>Ramichloridium</i> <i>cerophilum</i>	LSVM 1176	<i>Citrus grandis</i>	China	2014	Neg	Neg	Neg	-	-	-	MG956802
<i>Septoria cucubali</i>	LSVM 1208	<i>Passiflora edulis</i>	Guadeloupe	2015	Neg	Neg	Neg	-	-	-	MG956803
<i>Stenella musae</i>	LSVM 535	<i>Musa</i> sp.	Guadeloupe	2012	Neg	Neg	Neg	-	-	-	MG956804
<i>Xylaria acuta</i>	C- Ethiop-16	<i>Citrus aurantiifolia</i>	Ethiopia	2015	Neg	Neg	Neg	-	-	-	MG956805
<i>Xanthomonas</i> <i>alfalfae</i> subsp. <i>citrumelonis</i>	CFBP3138	<i>Citrus</i> sp.	Unknown	2016	Neg	Neg	Neg	-	-	-	
<i>X. alfalfae</i> subsp. <i>citrumelonis</i>	JJ238-28	<i>Citrus</i> sp.	Unknown	2016	Neg	Neg	Neg	-	-	-	
<i>X. citri</i> pv. <i>bilvae</i>	NCPBP3213	<i>Citrus</i> sp.	Unknown	2016	Neg	Neg	Neg	-	-	-	
<i>X. citri</i> pv. <i>citri</i>	CFBP2525	<i>Citrus</i> sp.	Unknown	2016	Neg	Neg	Neg	-	-	-	
<i>Zasmidium</i> <i>citri-griseum</i>	17NCC3	<i>Citrus sinensis</i>	Spain	2008	Neg	Neg	Neg	-	-	-	
<i>Zasmidium</i> <i>citri-griseum</i>	19NCC3	<i>Citrus sinensis</i>	Spain	2011	Neg	Neg	Neg	-	-	-	

Matrix A microtube, and ground with 400 µl of API lysis buffer. DNA was extracted and purified as described above for fungal tissue using the DNeasy Plant mini kit. The raw DNA extract from fruit or leaf tissue was directly used as template for PCR and real-time PCR (qPCR) and stored at -30°C until testing. The second halves of citrus lesions were surface sterilized with 70% ethanol, cut into small pieces, and plated on Malt extract agar supplemented with 100 ppm chloramphenicol, then incubated for 10 days at 25°C with a 12-h day/night photoperiod. After morphological observation, DNA from the colonies resembling *Elsinoë* spp. or *Pseudocercospora* spp. was extracted as described in the above section, and was tested by the quadruplex qPCR. For eight samples randomly selected, the colonies similar to the target species were molecularly identified by ITS sequencing. The ITS 1 and 2 region of the rDNA region was amplified by PCR using ITS1 and ITS4 primer pair, according to White et al. (1990) and sequenced using the same primers (Table 2). The sequence of each amplicon was compared by BLAST analysis to reference sequences available on the GenBank database.

Statistical analyses. Statistical analysis was performed with STATISTICA and R version 3.1.3. The normal distribution of the Ct values was tested by Shapiro-Wilks normality test. Data were subjected to the analysis of variance ANOVA and pairwise differences between the mean Ct values were compared using Tukey's honestly significant difference test. Differences were considered to be statistically significant at $P < 0.05$. Analysis of covariance (ANCOVA) was performed to compare regression lines and intercepts of the standard curves generated with the different background matrices (TE, lemon, and orange). Chi-squared tests for homogeneity were performed to compare the analysis of naturally infected citrus samples by plating, conventional PCR, and qPCR methods.

Results

Specificity assessment of the assays. Based on the 10 partial *MS204* gene sequences generated during this study (Table 2), polymorphic regions were identified enabling the successful design of

Table 2. GenBank accession numbers of the sequences used in the development of Conventional PCR and quadruplex qPCR assays for *Elsinoë fawcettii*, *E. australis*, and *Pseudocercospora angolensis*

Species (isolate)	Target gene	Accession number	Source
<i>Elsinoë fawcettii</i> (CBS 232.64)	<i>MS204</i>	MG891734	This study
<i>E. fawcettii</i> (EFAW-MUR4)	<i>MS204</i>	MG891735	This study
<i>E. fawcettii</i> (PK-MUR-L1)	<i>MS204</i>	MG891736	This study
<i>E. fawcettii</i> (USA-RUS-15)	<i>MS204</i>	MG891737	This study
<i>E. fawcettii</i> (Citr1)	<i>MS204</i>	MG891738	This study
<i>E. australis</i> (CBS 229.64)	<i>MS204</i>	MG891739	This study
<i>E. australis</i> (CBS 313.32)	<i>MS204</i>	MG891740	This study
<i>E. veneta</i> (CBS 150.27)	<i>MS204</i>	MG891741	This study
<i>E. centrolobi</i> (CBS 222.50)	<i>MS204</i>	MG891742	This study
<i>Pseudocercospora angolensis</i> (CBS 112748)	<i>MS204</i>	MG891743	This study
<i>P. angolensis</i> (CBS 112748)	<i>Tef-1α</i>	JX901669.1	Quaedvlieg et al. (2012)
<i>P. angolensis</i> (CBS 149.53)	<i>Tef-1α</i>	JQ324988.1	Crous et al. (2013)
<i>P. angolensis</i> (CBS 112933)	<i>Tef-1α</i>	GU384548.1	Crous et al. (2013)

Table 3. List of primers and probes for conventional and quadruplex real time PCR assays

Target (DNA region)	PCR test	Name ^a	Sequence (5'-3')	Position ^b	Size (bp)	Tm (°C)	Amplicon size
<i>Elsinoë fawcettii</i> (<i>MS204</i>)	Conventional	faw-Conv-F	ACCCTGTTCTATATCTTTTCCGAG	611-634	24	57.8	222 bp
		faw-Conv-R	TCTCGAGAAGTGAGCAGGAC	813-832	20	58.8	
	Real time	E.faw-F	AGAGCCGGAAGGAGCATG	750-768	19	59.8	66 bp
		E.faw-R	GACTGCGTGGAAAGAAAATTGC	794-815	22	60.9	
<i>E. australis</i> (<i>MS204</i>)	Conventional	FAM-TGGGGCTGTCATCCGTCAGT-BHQ1		770-789	20	64.3	
		aust-Conv-F	TCAGCCCATGTCCGGTATTG	75-94	20	59.8	569 bp
	Real time	aust-Conv-R	ATGGAGTATAAGGGTATGGGTCGA	620-643	24	60.2	
		E.aust-F	TCAGCCCATGTCCGGTATTG	75-94	20	59.8	90 bp
		E.aust-R	CGCACTGATTTATCTACCAATTGG	142-164	23	57.6	
		E.aust-P	ROX-CGCGCTCGAACTGCACATGGT-BHQ2	98-118	21	67.2	
<i>Pseudocercospora angolensis</i> (<i>Tef-1α</i>)	Conventional	ango-Conv-F	TATCACCGCCGTCACATCATC	22-44	21	60.3	213 bp
		ango-Conv-R	GCTGTATAAACTGGCGATAGC	217-236	20	57.3	
	Real time	P.ango-F	CCACACATTCCTCTGCTCCT	177-196	20	59.4	77 bp
		P.ango-R	AGGCATCGTATCAGCAAGCT	234-253	20	59.5	
		P.ango-P	CY5-TGAGCATCGACAACAACACGCT-BHQ2	198-219	22	63.3	
Plant/fungus ^c (18S rDNA)	Real time	18S uni-F	GCAAGGCTGAACTTAAAGGAA	1,105-1,126	22	59.9	150 bp
		18S uni-R	CCACCACCATAGAATCAAGA	1,234-1,254	21	59.8	
		18S uni-P	JOE-ACGGAAGGGCACCACCAGGAGT-BHQ1	1,130-1,151	22	70.3	
Fungus- universal ^d (ITS region)	Conventional	ITS1-F	TCCGTAGGTGAACCTGCGG	1769-1787	19	68.4	450-920 bp
		ITS4-R	TCCTCCGCTTATTGATATGC	40-61	20	61.5	

^a F for forward primer, R for reverse primer, and P for hydrolysis probe.

^b Position of the primer or probe considering as reference sequences GenBank accessions; MG891734 for *E. fawcettii*, MG891739 for *E. australis*, and JX901669 for *P. angolensis*.

^c Ioos et al. 2009.

^d White et al. 1990.

primer and probe combinations specific to *E. fawcettii* and *E. australis*. In addition, a set of conventional and real-time PCR oligonucleotides was designed targeting the *Tef-1a* gene to detect *P. angolensis* specifically. The DNA target regions for each primer and probe were 100% conserved within the target species. Both the conventional PCR and multiplex qPCR assays proved to be highly specific when tested against a wide panel of DNA target and nontarget species. Similar results were obtained in monoplex real-time PCR assays with each primer/probe combination when performed individually (data not shown). Low Ct values or a clear sharp band of the expected size were generated for all the DNA extracts corresponding to the target species, whereas no signal was obtained with any of the nontarget taxa. In addition, no cross-reaction with DNA extracted from citrus fruit was observed (Table 1).

Assessment of performance criteria. The conventional PCR test successfully yielded 100% repeatable positive results with as little as 100 pg μL^{-1} of target DNA per reaction for both *E. fawcettii* and *E. australis*, whereas 10 pg μL^{-1} was sufficient to successfully amplify *P. angolensis* target DNA. The multiplex qPCR assay successfully yielded repeatable positive results with as low as 243, 241, and 242 pc μL^{-1} for *E. fawcettii*, *E. australis*, and *P. angolensis*, respectively. Therefore, these concentrations were considered as the LOD for each test and target species. They were subsequently used as the reference quantity for the experiments addressing repeatability, reproducibility, and robustness of the qPCR assay. The dynamic range of the qPCR reaction was determined from the standard curves using serially diluted pDNA of *E. fawcettii*, *E. australis*, and *P. angolensis*. A high level of sensitivity was achieved over five orders of magnitude of pDNA concentrations that exhibited a linear dynamic range of amplification (Fig. 1). The correlation coefficients between the Ct values and the amount of pDNA were high for the three targets tested singly or in mixtures, also when diluted in different matrices. Generally, the R^2 showed that all Ct values followed a linear regression when plotted against the target concentration. The R^2 values for *E. fawcettii* ranged between 0.9881 and 0.9999, while the PCR efficiency (% E) calculated from the slope ranged between 98.50 and 102.0%. For *E. australis*, R^2 values ranged from 0.9975 to 0.9999 with the % E range between 92.09 and 97.49%, and similarly R^2 values ranged between 0.9900 and 0.9998 and the % E between 95.46 and 98.72% for *P. angolensis*. No significant effect was noted on the PCR efficacy and reaction dynamics of any target tested individually or mixed with the other two targets. The standard curves slopes and intercepts from plasmid DNA diluted in the different background matrices, and with or without the presence of the other species' genomic DNA, were not statistically different in any of the studied cases. For *E. fawcettii*, *E. australis*, and *P. angolensis* PCR reactions, neither slopes of the regression lines ($F = 0.026$, $P = 0.974$; $F = 0.009$, $P = 0.991$; $F = 0.032$, $P = 0.968$, respectively), nor y-intercepts ($F = 0.208$, $P = 0.812$; $F = 0.071$, $P = 0.931$; $F = 0.148$, $P = 0.863$, respectively) were affected (Fig. 1).

The intra-assay and interassay CVs indicated that the multiplex qPCR method was highly repeatable and reproducible. As shown in Table 4, the mean Ct value coefficient of variation (CV) for *E. fawcettii* ranged from 0.49 to 1.19% for the intra-assay and 0.55 to 1.60% for the interassays. The CVs for *E. australis* intra-assays and interassays ranged from 0.96 to 1.74 and 1.23 to 3.96%, respectively. For *P. angolensis*, both the interassay and intra-assay CVs were at the same level, with 0.49 to 1.05% and 1.26 to 2.47%, respectively.

The transferability of the qPCR assay was assessed using another real-time PCR platform (Fig. 2). Significantly higher Ct values ($P < 0.0001$) were obtained using the LightCycler 480 thermal cycler (96-well thermal block) compared with Rotor-Gene platform (72-well rotor). The difference of Ct values ranged from 2.9 to 9.1, and was the largest when working close to the limit of detection. However, these quantitative differences did not affect the qualitative final results, positive versus negative detection. In addition, the test specificity was not affected as no cross-reactions with the closely related, nontarget species was observed, i.e., *E. fawcettii* versus *E. australis* and *P. angolensis* versus *P. fijiensis*, regardless of the qPCR platform used.

A thorough evaluation of the quadruplex assay robustness was carried out using different concentrations and types of target DNA to verify the effect on sensitivity. To verify the effect on specificity, gDNA from a closely related but nontarget species was included for testing. For each of the three targets, the mean Ct values were sometimes significantly affected by a deliberate $\pm 10\%$ variation of the reaction, the template DNA volumes, or also by a $\pm 2^\circ\text{C}$ of the hybridization temperature (Fig. 3). These effects were observed regardless of the quantity and type of target DNA, but followed no obvious systematic pattern. Under our experimental conditions, artificially modifying the PCR reaction parameters never increased the Ct values by more than 1.8. (Hybridization temperature increased by 2°C with *E. fawcettii* gDNA as a template.) The maximum mean Ct values that were reached by modifying the qPCR parameters were always less than 40, with 26.7, 29.5, and 27.7, for *E. fawcettii*, *E. australis*, and *P. angolensis*. These results meant that each pathogen would still be detected, even at the lowest concentration levels. In addition, for

Fig. 1. Standard curves assessed with a 10-fold serial dilution of the *Elsinoë fawcettii* (A), *E. australis* (B), and *Pseudocercospora angolensis* (C) plasmidic DNA positive controls. The plasmidic DNA standards were diluted in 1× Tris-EDTA (TE) buffer and tested singly (black diamond), or in mix (black square); or diluted in a background of lemon DNA (black triangle) or in a background of orange DNA (gray cross) and tested in a multiplex assay. The mean Ct values are calculated with 3 replicates.

each primer set, no amplification was observed with DNA from the three nontarget species tested (no Ct value was generated before the completion of 40 PCR cycles), e.g., *E. australis*, *P. angolensis*, or *P. fijiensis* DNA was not amplified with the *E. fawcettii*-specific test, and vice versa. No cross-reactions were observed between any of the three target species primer-probe combinations and with DNA from the two other target species, even under the least stringent conditions such as a 10% reduction of reaction volume, a 10% increase of template DNA volume, or -2°C decrease of hybridization temperature (data not shown).

Analysis of naturally infected citrus samples. A total of 176 infected citrus samples collected from Ethiopia, Cameroon, the United States, and Australia were tested by the quadruplex qPCR, and 104 of them were also analyzed by agar plating followed by molecular analysis of the fungal colonies. Real-time PCR and plating analysis yielded concordant results (positive or negative) in 8.6% of the samples for *E. fawcettii* and 33.6% of the samples for *P. angolensis* (Table 5). *E. australis* was not detected by either technique. Homogeneity tests revealed that real-time PCR yielded significantly more positive results than isolation and molecular identification, for both *E. fawcettii* ($\chi^2 = 13.31$, $P = 0.0002$), and for *P. angolensis* ($\chi^2 = 26.39$, $P = 2.85 \times 10^{-7}$). After plating, only the fungal colonies with slow growth and typical colony morphology of either *E. fawcettii* or *P. angolensis* were selected for molecular identification. Four samples were rated as negative for all three targeted species, by direct

qPCR and by plating. The mean Ct values generated for the 18S uni test were low and ranged from 9.01 to 12.69, confirming that the DNA extracts were amplifiable, and that the lesions taken from these samples may have been caused by another disease, physiological disorder, or physical damage.

From eight citrus samples randomly selected, the molecular identification of the isolate recovered from the plating analysis by ITS sequencing confirmed the identity of the target species, i.e., four isolates were assigned to *P. angolensis* and four isolates to *E. fawcettii*. The sequencing results were in complete agreement with the qPCR test conducted on the same lesions, thus supporting the experimental specificity of the qPCR assay.

Discussion

In this work, a new quadruplex qPCR assay was developed to detect in a single reaction three important citrus pathogens: *E. fawcettii*, *E. australis*, and *P. angolensis*. These pathogens are not known to occur in the EU-Mediterranean citrus-producing areas and could potentially enter and establish through infected fruit. They are considered to be quarantine pathogens in many countries, while in EU they are regulated as harmful organisms and are listed in Council Directive 2000/29/EC. According to EFSA PLH Panel (2017a), around 71 to 79% of the total volume of citrus fruit imported from 2011 to 2015 into EU member states originated from countries where *E. fawcettii* in particular is present. Moreover, 64 interception cases of *Elsinoë*

Table 4. Intra-assay and interassay experiments carried out to assess the repeatability and reproducibility of the quadruplex qPCR

Target	Concentration	Intra-assay		Interassay	
		Mean Ct $\pm$ SD ^a	CV (%)	Mean Ct $\pm$ SD	CV (%)
<i>Elsinoë fawcettii</i>	1000 $\times$ LOD ^b	18.79 $\pm$ 0.19	0.99	18.93 $\pm$ 0.21	1.11
	100 $\times$ LOD	22.05 $\pm$ 0.11	0.49	22.24 $\pm$ 0.24	1.08
	10 $\times$ LOD	25.38 $\pm$ 0.25	1.00	26.00 $\pm$ 0.14	0.55
	0.1 ng μl^{-1} gDNA	21.42 $\pm$ 0.25	1.19	21.50 $\pm$ 0.34	1.60
<i>E. australis</i>	1000 $\times$ LOD	19.58 $\pm$ 0.29	1.50	20.81 $\pm$ 0.54	2.60
	100 $\times$ LOD	22.66 $\pm$ 0.22	0.96	24.53 $\pm$ 0.30	1.23
	10 $\times$ LOD	25.60 $\pm$ 0.45	1.74	28.10 $\pm$ 0.77	2.76
	0.1 ng μl^{-1} gDNA	20.94 $\pm$ 0.25	1.18	22.70 $\pm$ 0.90	3.96
<i>Pseudocercospora angolensis</i>	1000 $\times$ LOD	19.23 $\pm$ 0.20	1.05	19.60 $\pm$ 0.25	1.26
	100 $\times$ LOD	22.57 $\pm$ 0.11	0.49	23.20 $\pm$ 0.34	1.47
	10 $\times$ LOD	26.00 $\pm$ 0.18	0.68	27.10 $\pm$ 0.67	2.47
	0.1 ng μl^{-1} gDNA	23.38 $\pm$ 0.21	0.89	24.10 $\pm$ 0.37	1.55

^a Mean Ct value and standard deviation were calculated with 10 replicates.

^b LOD (limit of detection) corresponds to 243, 241, and 242 of pc μl^{-1} for *E. fawcettii*, *E. australis*, and *P. angolensis*, respectively.

Fig. 2. Assessment of the transferability of the qPCR assay using two real-time PCR platforms by testing different levels of target pDNA (10, 100, 100 $\times$ LOD) and gDNA (1 ng/ μl). The values are the mean Ct values $\pm$ standard deviation ($n = 10$). Significantly differing mean Ct values are indicated by * (P value < 0.0001). No amplification of nontarget species DNA was obtained.

spp. on citrus were recorded from 2001 to 2017. For these reasons, the recent pest categorization performed by EFSA panel concluded that: (i) *E. fawcettii* and *E. australis* meet all the criteria to be considered as potential EU quarantine pests; (ii) since the EU legislation refers to citrus plants, the intraspecific diversity within each of those species does not affect their categorization, and (iii) the new species *E. citricola* described by Fan et al. (2017) is not considered in this pest categorization for the lack of studies on its biology, host range, and pathogenicity. Therefore, the phytosanitary measures must be strengthened to mitigate the risk of those pests.

Symptoms caused by *E. fawcettii* and *E. australis* do not enable the distinction between the two species, and some of the scab symptoms

may be similar to defects like wind scar. To confirm the occurrence of *E. fawcettii*, *E. australis*, and *P. angolensis* on traded plant material is of paramount importance to prevent the introduction of these fungi into disease-free areas. Visual inspection and plating methods are time-consuming and unreliable with older lesions but also require considerable expertise for species-level identification. PCR-based techniques can offer an alternative tool for rapid, accurate, and cost-effective detection and identification of individual species. Here, we have developed molecular tools that enable the detection of three pathogens at the same time with a turnaround time for analysis reduced to half-a-working day for qPCR and a working day for conventional PCR. This allows for the smooth and rapid transit of

Fig. 3. Robustness of the quadruplex qPCR assay assessed with plasmidic DNA controls set at 10× LOD (left-hand side) and 100× LOD (center) concentrations and 0.5 ng µl⁻¹ gDNA (right-hand side) diluted in 1× Tris-EDTA buffer for *Elsinoë fawcettii* (A), *E. australis* (B), and *Pseudocercospora angolensis* (C). LOD corresponds to 243, 241, and 242 of pc µl⁻¹ for *E. fawcettii*, *E. australis*, and *P. angolensis*, respectively. The values are the mean Ct value ± standard deviation, obtained with 10 replicates. Mean Ct values with the same letters are not different according to Tukey's honestly significant difference.

Table 5. Naturally infected citrus samples tested for the presence of *Elsinoë fawcettii* (Ef), *E. australis* (Ea), and *Pseudocercospora angolensis* (Pa) by direct in planta quadruplex qPCR, or by plating followed by quadruplex real-time PCR carried out on the fungal strains isolated. Plating was attempted on samples with a sufficiently large lesion to be cut in two homogenous halves.

Citrus species	Country	Organ	(Nb. positive /Nb. of samples) qPCR results with DNA from citrus tissues			Plating	(Nb. positive /Nb. of samples) qPCR results with DNA from fungal culture		
			Ef	Ea	Pa		Ef	Ea	Pa
<i>Citrus × aurantiifolia</i>	Ethiopia	Leaves	0/4	0/4	4/4	4/4	—	—	2/4
<i>Citrus × paradisi</i>	Cameroon	Fruit	0/42	0/42	42/42	36/42	—	—	19/36
<i>Citrus sinensis</i>	Cameroon	Fruit	0/30	0/30	30/30	23/30	—	—	14/23
<i>Citrus reticulata</i> × <i>sinensis</i> Temple	Florida	Leaves	6/8	0/8	0/8	2/8	1/2	—	—
<i>Citrus reticulata</i> × <i>sinensis</i> Temple	Florida	Fruit	24/24	0/24	0/24	19/24	6/19	—	—
<i>Citrus reticulata</i> Blanco	Australia	Fruit	8/8	0/8	0/8	3/8	1/3	—	—
<i>Citrus limon</i>	Australia	Fruit	32/35	0/35	0/35	16/35	1/16	—	—
<i>Citrus reticulata</i>	Australia	Leaves	25/25	0/25	0/25	—	—	—	—

citrus propagation material or citrus fruit. The assays were used subsequently to determine whether the tested citrus tissues were infected by at least one of the three targeted pathogens.

To guarantee adequate specificity and sensitivity of PCR-based methods for diagnostic purposes, it is critical to choose the proper genetic marker for each target. Designing successful primer and probe requires that the region of interest be unique to the target species and conserved among its population (Rodríguez et al. 2015). Developing specific diagnostic primers and probes for *E. fawcettii* and *E. australis* was hampered by difficulties in identifying specific features among their pathotypes. None of the screened loci, ITS region of rDNA, *Tefl*- α , 28S large subunit ribosomal RNA gene, and β -tubulin was suitable for design of specific primers and probes. There was either low variation between *E. fawcettii* and *E. australis* or high variability within the same species populations. Similar results were found by Fan et al. (2017) in their study on the phylogenetic relationships among *Elsinoë* species. Differentiating between *Elsinoë* species based on a single genetic locus is a vexing issue; however, when ITS, LSU, *rpb2*, and *Tefl*- α sequence data were combined, *E. fawcettii* and *E. australis* were clearly separated (Fan et al. 2017). The newly described marker *MS204* (Walker et al. 2012) chosen for primers/probe design in the *E. fawcettii* and *E. australis* assays was unique with the presence of variable regions appropriate for differentiating species of *Elsinoë*. In addition, sequence analysis of *MS204* from different isolates of the same species revealed the absence of interspecific polymorphism. *MS204* is a single-copy protein-coding gene that codes for a guanine nucleotide-binding protein subunit beta-like protein (Walker et al. 2012). The marker is among a category of binding proteins which were determined by Aguileta et al. (2008) as the best performing genes for fungal phylogenetics. Moreover *MS204* was determined to be a superior marker compared with ITS and β -tubulin for species-level relationships in another class of ascomycetes (Walker et al. 2012). For *P. angolensis*, we designed the specific primers and probe based on the sequence of *Tefl*- α . This gene is extensively used in *Pseudocercospora* genus phylogeny, because it is found to be highly informative at the species level (Crous et al. 2013). Based on the same polymorphic DNA regions, three primer pairs were also designed for use in species-specific conventional PCR.

The quadruplex qPCR was fully validated in the framework of this study. It proved to be highly and consistently specific (100%), since no cross-reaction was observed either with genetically related nontarget species, or with all the tested fungal species that were associated with citrus tissues. In the present work, 46 nontarget DNA templates representing 24 other pathogenic and saprophytic fungi or bacteria as well as citrus DNA were tested. In the case of *E. fawcettii*, another qPCR method for detection was described previously by Hou et al. (2013), but *E. australis* was not included to test the specificity. Moreover, this qPCR assay is based on SYBR green chemistry, which has several intrinsic limitations for detection purposes (Smith and Osborn 2009). Real-time PCR based on hydrolysis probes has not been reported for detecting any of the three pathogens and has several benefits including enhanced specificity over SYBR Green and the ability to multiplex primers and probes. Similarly, the primer pairs developed for conventional PCR exhibited high specificity when tested with the isolates of the other species of concern and other genera. A recent study by Fan et al. (2017) described *Elsinoë citricola* sp. nov. causing *Citrus limon* scab. This new taxon cannot be distinguished from *E. fawcettii* based on ITS or LSU rDNA sequences. We did not have access to any *E. citricola* isolate in the course of our study to determine if its DNA cross-reacts with the *E. fawcettii*-specific tests we developed. This new species has only been described in Brazil up to now, and may have been confused with *E. fawcettii* for many years at least in this country. Therefore, although a large collection of pathogens associated to citrus has been included in this work, we advocate continuous and frequent verification of the specificity of the assays, as we certainly have not covered the entire biological diversity of fungi occurring on this plant.

One of the most critical performance criteria for diagnostic methods is the lowest amount of the target which can be detected with

acceptable reproducibility and accuracy (Forootan et al. 2017). The detection limits of the quadruplex assay were 243, 241, and 242 pc μL^{-1} of *E. fawcettii*, *E. australis*, and *P. angolensis* target DNA, respectively, which was comparable to LOD ranges reported in a similar study targeting a single copy gene in other fungal pathogens by a multiplex approach (Guinet et al. 2016). The sensitivity of the conventional PCR assay was shown to be acceptable with a LOD of 100 pg μL^{-1} of gDNA for both *E. fawcettii* and *E. australis* and 10 pg μL^{-1} for *P. angolensis*. A direct comparison of the sensitivity of our conventional PCR assays with the only other available tests developed by Hyun et al. (2007) for *E. fawcettii* and *E. australis* is difficult. The LOD of their test was not well defined, although it was mentioned that the method could detect the pathogens from a single lesion on fruit. These values of LOD could explain the fact that both qPCR and conventional PCR assays were developed using single-copy coding protein genes. Although the sensitivity of conventional PCR assay proved to be generally lower than qPCR (Ioos et al. 2010), these tools may still be of great interest in diagnostic laboratories where real-time machines are not available.

The most reliable and robust approach to estimate the amplification efficiency and the linearity for each target in a multiplex method is by constructing standard curves. Here, the standard curves obtained for the quadruplex assay had slopes that ranged from -3.272 to -3.527 with R^2 values > 0.9985 and PCR efficiencies that ranged from 92 to 102%. Although the optimum values for PCR efficiencies are not stated in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009), these values are in line with the guidelines described by Broeders et al. (2014) for validation of qualitative real-time PCR methods. Detection methods are considered robust when the whole validation process has been appropriately carried out. The minimum performance criteria of a test to fulfill the requirements of EPPO standard PM 7/98 for routine use (EPPO Bull. 2010) should include assessment of the repeatability and reproducibility. The quadruplex qPCR assay developed in this study was demonstrated to be highly repeatable and reproducible as shown by the low CVs for the mean Ct values in the intra-assays and interassays tests. The robustness reflects the capability of a method to withstand slight changes in experimental conditions with respect to a standard protocol (Vander Heyden et al. 2001). The data showed that under the test conditions, 10% variations of PCR reaction or of DNA template volumes, as well as a $\pm 2^\circ\text{C}$ variation of the annealing temperature did not qualitatively affect the results in term of sensitivity and specificity. The quadruplex assay was shown to be robust and was able to withstand the slight pipetting errors or temperature drift of a thermal cycler without compromising the accuracy of the results.

Transferability of a method to another laboratory requires careful understanding of the factors involved in generating reproducible results, such as those that might be introduced by applying the method using different types of real-time PCR machine. In this study, we used two PCR platforms available in the lab to test if the quadruplex assay and found that it could easily be transferred to different machines. Although the results showed that the type of qPCR instrument used had a significant effect on the assay performance, nevertheless, changing the PCR platform did not affect the specificity and sensitivity of the quadruplex results confirming transferability of the assay. Differences in mean Ct values are not surprising and most likely are due to the particular algorithms used to compute the Ct values, also the specific hardware and software settings in each instrument. The transfer of any protocol to a new qPCR instrument should start with running a calibration curve using standard samples, such as plasmidic positive controls. This would give a good idea of the detection threshold that can be confidently achieved under the new experimental conditions, and help to compare the results between and within labs.

The reliability of the quadruplex qPCR assay was further validated by detecting the targets on 176 samples of naturally infected citrus tissues. The samples were collected from three different continents and represent a wide range of *Citrus* species including *Citrus aurantiifolia*, *C. reticulata*, *C. paradisi*, *C. sinensis*, *C. limon*, and

C. reticulata Blanco (satsuma hybrid). The quadruplex qPCR and plating assays detected *E. fawcettii* and *P. angolensis* from symptomatic leaves and fruit. In this study, although *E. australis* could infect all the tested citrus species, we could not find any cases of *E. australis* infection from the tested samples. A plausible explanation for these results is that *E. australis* has limited geographical distribution, mainly South America, whereas *E. fawcettii* is widely distributed. The tested samples originated from countries where *E. australis* is known to be absent (Ethiopia and Cameroon), Florida (where *E. australis* is present but with limited occurrence), and Australia (where *E. australis* is present but with restricted distribution). The detection of *E. australis* would have been more likely in fruits sampled in countries like Argentina or Brazil where this species is more common, but unfortunately, we could not have access to diseased fruits from these countries during the course of our study. The significant difference in number of positive results by plating and quadruplex methods highlights the complications in isolating these pathogens. *E. fawcettii*, *E. australis*, and *P. angolensis* are slow-growing fungi, and any faster growing fungi may easily over produce colonies. In many cases, *Fusarium* and *Colletotrichum* spp. were isolated and identified by the morphological characteristics of their spores. These two genera grow faster than *Elsinoë* and *Pseudocercospora* and might have out competed the target fungi on the plates. As shown by Tan et al. (1996), isolation of *Elsinoë* spp. might have been improved by the use of a semi-selective medium (Whiteside 1986), but unfortunately information regarding its effect on *P. angolensis* growth is not available.

These results indicate that the quadruplex qPCR offers better sensitivity to *Pseudocercospora* leaf and fruit spot and scab pathogens than the traditional plating method using a widely used isolation medium. Thus, laboratories using only isolation techniques to determine if a quarantine pathogen is present may be missing up to 65% of positive cases, considering that the plating method in our case detected only 40 positive results of 104 tested samples. The results showed that five of 176 tested samples were negative for all three targeted species, regardless of the technique used. This could be explained by the fact that symptoms induced by these pathogens may sometimes be confused with other diseases. During the course of this study, we experienced a case where all the samples received for testing which were diagnosed as potentially infected with citrus scab diseases by the local extension service, turned out to be affected by citrus canker (*X. citri* subsp. *citri*).

In conclusion, this study developed species-specific primers and probes for the detection and identification of three fungal pathogens of citrus. The assay was developed for fruit, but it could easily be adapted to testing propagation material such as budwood, providing that a suitable DNA extraction protocol is optimized. The tests can be used either individually in conventional PCR or in a multiplex qPCR assay, depending on the level of equipment available in the testing laboratory, and may be of great interest in phytosanitary inspections. Isolation of these pathogens by plating is time-consuming and requires confirming the identity through sequence analysis. The quadruplex qPCR described here is fast and capable of detecting the three pathogens without reliance on another diagnostic approach for confirmation. The assay was also designed to include an internal DNA quality control under multiplexing conditions to assess the possible inhibition of qPCR, and thereby identifying potential false negative samples (Ioos et al. 2009). However, in light of the recent finding of a cryptic species confused with *E. fawcettii*, i.e., *E. citricola*, the first report of any of the three target pathogens in countries where it is not known to occur should be confirmed by isolating the fungus in pure culture for morphological and molecular characterization.

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