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Development of a Real-Time PCR Assay for Early Detection of the Eucalyptus Pathogen *Quambalaria eucalypti*

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Abstract: *Quambalaria eucalypti* is a fungal pathogen that causes leaf spot, shoot blight, and stem canker on *Eucalyptus* spp. Early diagnosis of the disease is difficult, although symptoms are clear in the advanced phase. To enable rapid and sensitive screening of asymptomatic or latent-infected plant material for *Q. eucalypti*, a SYBR green-based real-time PCR assay targeting the partial histone-H3 region was developed. The assay demonstrated specificity for *Q. eucalypti*, not showing cross-reactivity with other *Quambalaria* species or other eucalyptus fungal pathogens tested. The primers developed in this study ensured high analytical sensitivity, allowing the detection of *Q. eucalypti* DNA concentrations as low as 10 fg DNA from asymptomatic plants. The robustness and efficacy of the assay was demonstrated by interlaboratory comparisons with similar results. This newly developed quantitative PCR assay can be used for more comprehensive epidemiological investigations, testing plant material in known *Q. eucalypti* distribution areas for early management strategies, or collecting data for resistance breeding programs.

1. Introduction

The genus *Eucalyptus* L'Hér., which includes about 900 species in the family Myrtaceae, is native to Australia and its neighbouring islands [1]. Most eucalypts plants are evergreen woody perennial shrubs and tall trees that grow rapidly, reaching heights of up to about 90 metres. Todays, eucalyptus plantations are the most widely planted broadleaf forests in the world, with the majority located in tropical and temperate regions. They are also widely used in afforestation programmes [2]. It is estimated that the total area of eucalyptus plantations worldwide exceeds 22.57 million hectares based on a survey conducted in 65 countries with extensive plantations [3]. Eucalyptus plants have been well known since ancient times for the numerous uses of their components (wood, fibres, cellulose, dyes, pulp, rubber, resin, essential oils) which are used in various sectors such as construction, pharmaceutical and plant protection [4].

Among the fungal diseases affecting eucalyptus forestry in various regions of the world are those caused by species of the genus *Quambalaria* (Quambalariaceae, Basidiomycota). Of particular relevance is *Quambalaria eucalypti* (M.J. Wingfield, Crous, & W.J. Swart) J.A. Simpson [5], which commonly infects eucalyptus plants in nurseries but also in plantations [6,7]. In particularly, the pathogen is known to cause significant productivity losses clonal cuttings of mini hedge plants used for vegetative propagation in nurseries [8].

This fungal species was originally described as *Sporothrix eucalypti* causing leaf spots and shoot dieback on a clone of *Eucalyptus grandis* in South Africa [5]. The same fungus was later associated with stem cankers in plantations of *Eucalyptus globulus* in Uruguay [9], while in Brazil it was reported to cause stem girdling on seedlings of *E. globulus* and leaf and shoot blight on ministumps of a hybrid of *Eucalyptus saligna* x *E. maidenii* [10]. It has since been recognized as a basidiomycete and placed in

the genus *Quambalaria* [11]. Disease caused by *Q. eucalypti* has subsequently been reported from Australia [12], Portugal [13], China [14] and, most recently, Indonesia [7].

$\qquad$*Q. eucalypti* is currently identified through the isolation of the fungus and species recognition via morphological examination and phylogenetic analysis by using both the ITS and LSU regions of ribosomal DNA. However, these techniques are laborious, costly and time-consuming. Quantitative PCR (qPCR) is one of the most widely used molecular methods, as it does not require post-amplification processing steps (e.g. gel electrophoresis) which are required in conventional endpoint PCR (cPCR), and allows a specific quantification of the target DNA in the sample [15]. As knowledge of the life cycle and epidemiology of *Q. eucalypti* in the host is still limited, early detection and quantification of this pathogen is crucial in order to plan an appropriate disease management strategy.

$\qquad$To date, no studies have been conducted to rapidly detect and quantify *Q. eucalypti* in eucalyptus plant tissues by using a molecular approach. Therefore, the aim of this study was to develop and validate a specific and sensitive real-time PCR assay for the early detection and quantification of *Q. eucalypti* in eucalyptus plants.

2. Materials and Methods

2.1. Fungal Isolates and Plant Materials

$\qquad$To validate the real-time PCR assay, 30 isolates of *Q. eucalypti* and 19 isolates of nontarget species from *Eucalyptus* spp. and various geographical regions were used (Table 1). The *Q. eucalypti* isolates from Brazil were obtained from leaves of *E. globulus* seedlings in two nurseries located in the states of Espírito Santo and Rio Grande do Sul in 2021 and 2022, respectively. Uruguayan isolates were obtained in 2004 from twigs with cankers of *E. globolus* in a plantation located in the west-littoral region. The non-target isolates included 4 other *Quambalaria* species, and 9 isolates from different species of genera associated with foliar diseases of *Eucalyptus* spp. The identifications of the *Quambalaria* isolates were confirmed by sequencing the ITS and LSU regions using primer pairs ITS1F-ITS4 [16,17] and LR0R-LR5 [18,19], respectively. Isolates from species of other genera were identified by ITS sequencing using the ITS5-ITS4 primer pair [17] and, for some of them, the beta-tubulin and elongation factor genes were also sequenced using the primers Bt2a and Bt2b [20], and EF1-728F [21] and EF2 [22], respectively. The isolates were maintained as monosporic cultures into tubes containing malt extract agar (MEA) and stored at 4±1°C.

Table 1. Fungal isolates examined in this study and results of specificity testing of the qPCR assay.

Species	Isolate[a]	Host	Origin	Ct[b] value ± SD[c]	Tm (°C)[d]
Quambalaria eucalypti	CBS 118844[e]	*Eucalyptus grandis*	South Africa	21.54±0.71	87.2
	CBS 119680[f]			21.30±0.52	87.0
	ITEF/1937			20.48±0.35	86.1
	ITEF/1999			20.43±0.14	86.8
	EGES-1	*Eucalyptus globulus*	Brazil	21.14±0.35	86.1
	EGES-2			20.87±0.62	87.1
	EGES-5			20.91±0.53	87.0
	EGES-10			22.07±0.26	86.0
	EGES-11			22.09±0.88	86.2
	EGES-13			21.30±0.13	87.0
	EGES-17			21.58±0.44	86.9
	EGES-19			22.43±0.46	86.1
	EGES-20			22.46±0.35	86.2

Species	Isolate	Host	Country		
	EGES-22			22.45±0.21	87.2
	RGS-1			21.41±0.57	86.3
	RGS-2			21.35±0.22	87.0
	RGS-3			21.47±0.31	87.2
	RGS-4			21.29±0.81	86.2
	RGS-5			20.93±0.73	87.0
	RGS-6			21.02±0,54	86.6
	SUL/01			21.27±0.52	86.7
	SUL/03			22.05±0.54	86.8
	UY198			21.24±0.31	86.3
	UY199			21.63±0.61	87.0
	MATY 4665		Uruguay	19.98±1.07	86.5
	MATY 4751			20.15±0.86	86.5
	MATY 4752			21.73±0.24	86.7
	MATY 5001			21.65±0.35	86.6
	3421-S			19.82±1.13	87.0
	3422-T			21.36±0.52	87.1
Quambalaria cyanescens	CBS 876.73	*Eucalyptus pauciflora*	Australia	n/a	n/a
	IMI 178848			n/a	n/a
	EU-PA			n/a	n/a
Quambalaria pitereka	CERC/03/08	*Eucalyptus* sp.	China	n/a	n/a
	CERC/04/08			n/a	n/a
	CERC/05/05			n/a	n/a
Quambalaria simpsonii	CBS 124772[f]	*Eucalyptus tintinnans*	Australia	n/a	n/a
	EU-TI3			n/a	n/a
Quambalaria tasmaniae	CBS 145602[a]	*Eucalyptus* sp.	Australia	n/a	n/a
	7608			n/a	n/a
Alternaria alternata	CYC-60	*Eucalyptus grandis*	Uruguay	n/a	n/a
Calonectria spathulata	CS11340	*Eucalyptus grandis*	Brazil	n/a	n/a
Colletotrichum boninense	MWJ-43	*Eucalyptus grandis*	South Africa	n/a	n/a
Cryphonectria havanensis	GSEG_95	*Eucalyptus grandis*	Brazil	n/a	n/a
Cylindrocladium candelabrum	11356	*Eucalyptus* sp.	Brazil	n/a	n/a
Cylindrocladium pteridis	IMI 354530	*Eucalyptus grandis*	Brazil	n/a	n/a
Neopestalotiopsis eucalyptorum	912/88	*Eucalyptus globulus*	Portugal	n/a	n/a

| *Phomopsis arnoldiae* | CR 345-96 | *Eucalyptus grandis* | Uruguay | n/a | n/a |
| *Pseudocercospora eucalyptorum* | BBR 5689 | *Eucalyptus globulus* | Spain | n/a | n/a |

[a] CBS = cultures from Westerdijk Fungal Biodiversity Institute, Netherlands; [b] Ct = cycle treshold; [c] SD = standard deviation; [d] melting temperature ± standard deviation; [e] ex-holotype; [f] ex-epitype; n/a = no amplification.

To confirm the sensitivity of the qPCR assay using infected plant material, three randomly selected *Q. eucalypti* isolates (i.e. CBS 118844, EGES-5, and UY198) were inoculated on 3-month-old potted *E. globulus* plants. For each isolate, five plants were inoculated by manually spraying a suspension of 1×10^6 ml^{-1} spores onto the surface that was previously pricked with a sterile 0.5 mm diameter syringe needle. The spores were obtained from 7-day-old fungal colonies grown on potato dextrose agar (PDA). Five control plants were sprayed with sterilised distilled water instead. Each plant was covered for 48 hours with a clear plastic bag to maintain high relative humidity. Each seedling was covered for 48 hours with a clear plastic bag and kept in the growth chamber at 30°C for 14 days under a 12-hour photoperiod and watered as needed. The material from the inoculated plants was then collected by sorting the samples into 4 different batches based on disease severity. An empirical symptom scale from 0 to 3 was assessed visually since the appearance of first symptoms as leaf spot or stem canker (0 = no symptoms, 1 = 1-25% leaf spot or stem canker, 2 = 26-75% leaf spot or stem canker, 3 = 76-100% shoot and leaf blight or clear signs of the pathogen. Symptomatic and non-symptomatic plant material was kept refrigerated until DNA extraction and then processed either separately or pooled and combined with healthy stem tissues sampled from the control plant in a 1:10 weight ratio.

2.2. Genomic DNA Extraction and Quantification

DNA extraction of fungal isolates was carried out from mycelium grown on PDA for 9 days at 24±1°C in Petri dishes, and that of eucalyptus plants from leaf and stem samples. Both fungal and plant samples were frozen in liquid nitrogen and ground in a a mortar with pestle. DNA was extracted from 50 mg and 100 mg of each mycelium and plant tissue sample respectively, by using the Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp, Canada) according to the manufacturer's instructions. Extract DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA) and adjusted to a concentration of 10 ng μl^{-1}. Genomic DNA with an A260/A280 ratio between 1.8 and 2.0 was used for PCR analyses.

2.3. Primer Design and cPCR Amplification

The Primer-Blast program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) [23] was used to design the specific primers for a histone-H3 gene selected as the target locus. The sequences of the primers were searched against the whole draft genome of *Q. eucalypti* isolate CBS 118844 (Genbank accession no. RRYC01000000) [24]. The expected PCR product size parameter was set at 150-400 bp, and the optimal melting temperature at 60±3°C. In silico specificity was verified using Primer-Blast, while the potential formation of dimers and hairpin structures was checked using the tool OligoAnalyzer (https://eu.idtdna.com/calc/analyzer). PCR primers were synthesised and supplied by Invitrogen.

The designed primer pair was tested for their ability to amplify the expected product and to confirm specificity by gradient endpoint cPCR using DNA samples from all fungal isolates listed in Table 1. The amplification reactions were performed on an Arktik thermocycler (Thermo Fischer Scientific, Waltham, MA) in a final volume of 25 μl, containing 12.5 μl of AccuPower PCR Premix (Bioneer, Alameda, CA), 1 μl of each primer (10 μM), 1 μl of DNA (10 ng μl^{-1}), and DNase-free water to make up the final volume. Cycling conditions were as follows: an initial preheat at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58, 59, 60, 61, or 62°C for 30 s, and extension at 72°C for 3 min, followed by a final extension at 72°C for 7 min. PCR products were detected on 2% agarose gel containing SYBR safe DNA gel stain (Life Technologies) in a Tris-borate-

EDTA buffer with lane markers and a 100-bp small fragment ladder (Fermentas). Amplicons were purified with ExoSAP-IT (Affymatrix Inc., Santa Clara, CA) and sequenced in both directions by an external service (Macrogen, Amsterdam, The Netherlands). All the chromatograms generated were checked for correct base calling using FinchTV v.1.4.0. The sequences were aligned to the target histone-H3 sequence of *Q. eucalypti* isolate CBS 118844 using Clustal 2.1 software to confirm the match and to detect for possible polymorphic bases.

A duplex PCR assay was also performed to reveal any false negatives that might have occurred due to PCR inhibition. The primers from this study and those of the cyclin-dependent kinase (CDK8) gene [25] were used to co-amplify both fungal and plant DNA in a single reaction, with the CDK8 (196 bp) serving as a plant internal control. The PCR reaction followed the same procedure as the cPCR, but with 1.5 µl of a DNA mixture from *Q. eucalypti* isolate CBS 118844 and eucalyptus leaf and stem used as the template. The DNA mixture was composed of 0.5 µl fungal DNA, 0.5 µl leaf DNA (100 ng µl^{-1}) and 0.5 µl stem DNA (100 ng µl^{-1}). Five 10-fold dilutions (10^{-2} to 10^{2}) of fungal DNA (10 ng µl^{-1}) were tested.

2.4. SYBR Green Real-Time PCR Optimization

To optimise the assay, the qPCR reactions were performed in a gradient using an iCycler iQ Real-Time PCR DetectionSystem (Bio-Rad) in a final volume of 25 µl, which contained 12.5 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 1 µl of each primer (8, 10 or 12 µM), 1 µl of DNA (10 ng µl^{-1}) from CBS 118844, and DNase-free water to make up the final volume. Thermal cycling parameters consisted of an initial preheating step for 5 min at 95°C followed by 40 cycles at 95°C for 30 s, annealing at 59, 60, 60.5, 61, 61.5, 62, or 62.5°C for 30 s, and 72°C for 30 s for data collection and real-time analysis; then, the set point temperature was increased after cycle 2 from 72 to 95°C for 30 s in 0.5°C increments to construct a melting curve and perform data collection and analysis.

2.5. Real-Time PCR Assay Specificity and Sensitivity

The qPCR assay's specificity was tested using DNA samples from *Q. eucalypti* isolates listed in Table 1. The real-time PCR reaction conditions and thermocycling settings were optimized as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C, and extension at 72°C for 30 s. The sensitivity of the qPCR assay was determined by testing six 10-fold dilutions (10^{0} to 10^{-5}) of DNA (100 pg µl^{-1}) from *Q. eucalypti* isolate CBS 118844 in sterile water or in DNA extracted from eucalyptus leaf (10 ng µl^{-1}) or stem (10 ng µl^{-1}). The sensitivity test was conducted in triplicate for each dilution and three real-time PCR runs were carried out for each sample using the previously mentioned thermocycle conditions and DNA templates. Standard curves were generated by graphing the mean cycle threshold (Ct) values on the y-axis and the concentration of fungal DNA on the x-axis. The efficiency of the PCR assay was determined using the following equation: $E = (10^{1/-S}) -1$ [26].

2.6. Interlaboratory Tests

The mean Ct values and standard deviation of the intra- and inter-assay results were used to calculate the coefficient of variation (CV). The CV for intra-assay repeatability was assessed by conducting ten technical replicates in the same reaction using four dilutions of CBS 118844 isolate DNA in water (10, 1, 0.1, and 0.01 pg µl^{-1}). The CV between assays was determined from the results of three independent blind panel experiments (three technical replicates in each assay) performed on different real-time PCR machines. The reproducibility test was performed by three distinct operators using the same series of dilutions of the DNA as described previously. Additionally, to validate the applicability of the qPCR assay, the test was also carried out in an external laboratory on a different real-time PCR instrument (i.e. StepOnePlus qPCR system, Applied Biosystems) using different set of reagents.

3. Results

3.1. Primer Design and cPCR Amplification

A primer pair was successfully developed for a histone-H3, which is used as a target locus in the genome of *Q. eucalypti* isolate CBS 118844. The designed specific primers (QEH3-F: 5'-CTTAGGACTTCTCGCCTCGG-3' and QEH3-R: 5'-CTGAGCTCCTCATCCGCAAG-3') amplify a DNA segment of 237 bp (Figure 1). The comparative sequence analysis, conducted on nucloeotide database of NCBI GenBank, showed that the designed primers are specific to *Q. eucalypti* and do not share any identical or similar sequences with those of other fungi.


```
Sequence (5'->3')
CTTAGGACTTCTCGCCTCGGAGACGACGAGCCAGGGCAATGTCCTTGGGCTGGATGGTGACACGCTTGGCG
TGGATGGCAGCCAAGTTGGTGTCCTCGAAGAGACTGACGAGGTATGCCTCGGCGGCCTCCTGAAGCGCAGC
AATGGCAGAGCTCTGGAAGCGGAGGTCGGTCTTGAAGTCCTGGGCAATCTCACGGACGAGACGCTGGAAGG
GGAGCTTGCGGATGAGGAGCTCAGTCGACTTCTGGTAGCGACGAATCTCACGAAGAGCGACGGTACCGGGC
TTGTAACGGTGAGGCTTCTTGACTGTTGCGTGGGTGGGCGAGGGCGGGGGGATGGATGGGTGATACAG
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Figure 1. The nucleotide sequence of histone-H3 of *Quambalaria eucalypti* and the flanking regions of the designed primers shown in the red box.

The QEH3-F/QEH3-R primer pair successfully generated an amplicon of the expected lenght for all *Q. eucalypti* isolates tested in this study using gradient end-point PCR. In contrast, the non-target isolates of other *Quambalaria* species and those of different genera were not amplified. Direct sequencing of the PCR products from these isolates showed 100% identity with the targeted histone-H3 region and no ambiguous nucleotide sites, demonstrating the specificity of the primers and the absence of polymorphisms.

The duplex PCR assay amplified both the *Q. eucalypti* histone-H3 and the eucalyptus CDK8 targets from mixed DNA samples of the fungus and plant tissues with no evidence of inhibition.

3.2. SYBR Green Real-Time PCR Optimization

The target was successfully amplified using a gradient real-time PCR with newly designed primers. At different annealing temperatures (59 to 62.5°C) slight variations in Ct values were observed. For further assay development, an annealing temperature of 60°C was selected. At this temperature, the lowest Ct values and detection curves with high relative fluorescence were obtained. By assaying different primer concentrations (8, 10 and 12 μM), it was observed that the 8 μM concentration resulted in lower Ct values. Therefore, a concentration of 8 μM was selected for subsequent assay validation.

3.3. Real-Time PCR Assay Specificity and Sensitivity

Likewise the cPCR, the new real-time PCR assay detected all 30 *Q. eucalypti* isolates without cross-reacting with any of the 19 non-target fungal isolates or plant DNA. The primers QEH3-F and QEH3-R generated a single absorbance intensity peak for all *Q. eucalypti* isolates. Melting curve analysis of the target DNA samples showed melting points ranging from 86.5 to 87.3. No rise in absorbance was observed for the non-target fungal isolates or the negative control (Table 1).

The assay's analytical sensitivity using a 10-fold serial dilution of *Q. eucalypti* DNA in water or in eucalyptus leaf or stem DNA revealed that the assay was capable of detecting pathogen DNA levels as low as 10 fg. The sensitivity test showed a linear curve response from 100 pg to 10 fg DNA (Figure 2) with PCR efficiencies of 96 and 97% for pathogen DNA diluted in water and plant tissue, respectively.
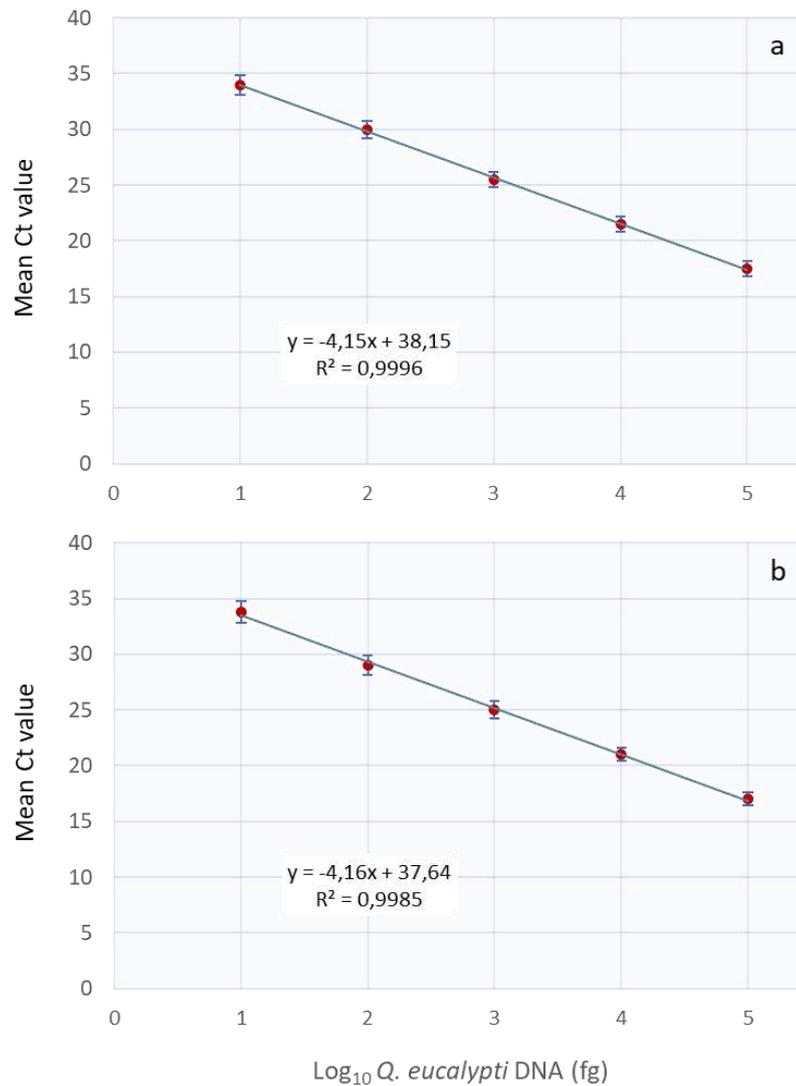
Figure 2. The Real-Time PCR assay standard curve of serial dilutions of *Quambalaria eucalypti* DNA in water (a) and in combination with eucalyptus DNA (b). A linear trend was observed for both dilution series from 10 to 10^5 fg DNA. The standard deviation is indicated by the vertical bars, while Ct corresponds to the quantification cycle.

The pathogen was successfully detected using DNA obtained from *Q. eucalypti*-infected leaves and stems artificially inoculated in eucalyptus seedlings. The Ct values obtained from the tissues of symptomatic plants were lower than those of asymptomatic plants, decreasing from 27.70 to 24.81 with increasing disease severity. However, amplification was also observed in asymptomatic samples with an average Ct value of 33, equivalent to about 18 fg of pathogen DNA. No significant differences in Ct values were observed between the *Q. eucalypti* isolates assayed (Table 2). The qPCR assay detected *Q. eucalypti* even in infected tissues mixed with healthy plant material in a ratio of 1:10. The fungus was not detected in DNA samples from non-inoculated eucalyptus plants.

Table 2. Quantitative PCR detection of *Quambalaria eucalypti* artificially inoculated in eucalyptus plants.

Isolate	Disease severity	Tm (°C)	Ct[a] value ± SD[b]	DNA conc. (fg) ± SD[b]
CBS 118844	0	87.2	33.10±0.02	16.48±2.40
	1	86.9	27.70±0.05	329.66±5.24
	2	87.3	26.75±0.03	558.45±11.07
	3	87.0	24.89±0.02	1,567.40±14.85
EGES-5	0	87.3	32.87±0.18	18.72±0.65
	1	86.8	27.43±0.27	382.94±5.18
	2	87.1	26.73±0.02	564.69±11.67
	3	86.9	24.95±0.03	1,516.08±12.36
UY198	0	87.0	32.84±0.26	19.03±0.15
	1	87.0	27.53±0.02	362.27±4.73
	2	86.9	26.71±0.04	570.99±10.45
	3	87.1	24.81±0.06	1,638.54±21.74

[a] Ct = cycle threshold; [b] SD = standard deviation.

3.4. Interlaboratory Tests

The test repeatability of the qPCR assay resulted in none or minimal Ct value variation. Furthermore, the intra-assay CV for the four tested dilutions of *Q. eucalypti* DNA remained consistently low. The assay's reproducibility testing showed only a slight difference in Ct values, with a low inter-assay CV (Table 3). Independent laboratory testing of the assay further confirmed its reproducibility.

Table 3. The test results for repeatability (intra-assay) and reproducibility (inter-assay) using four dilutions of DNA in water from *Quambalaria eucalypti* isolate CBS 118844.

DNA conc. (pg)	Ct[a] value ± SD[b] (intra-assay)	Ct[a] value ± SD[b] (inter-assay)			Coefficient of variance (%)	
		Operator 1	Operator 2	Operator 3	Intra-assay	Inter-assay
10	21.49±0.15	21.40±0.54	20.98±0.66	20.78±0.15	0.3	0.9
1	25.50±0.11	25.18±0.36	25.34±0.12	26.03±0.24	0.4	1.5
0.1	30.01±0.12	29.87±0.21	29.91±0.15	30.05±0.45	0.6	1.3
0.01	34.01±0.13	33.85±0.18	34.13±0.11	33.97±0.21	0.8	2.4

[a] Ct = cycle threshold; [b] SD = standard deviation.

4. Discussion

Among the molecular diagnostic techniques, qPCR is more specific, higher sensitive and less time-consuming than conventional endpoint PCR [27,28]. This technique enables quantification of pathogens and has been used for various fungal plant pathogens [29–31], including *Q. eucalypti* [32]. Duong et al. (2022) developed a qPCR assay for *Q. eucalypti* using the multi-copy ITS region, but it has not been tested on infected host tissue. In this study, a SYBR green real-time PCR assay for the detection and quantification of *Q. eucalypti* in eucalyptus plants was successfully developed. The use of a qPCR detection method based on single-copy genes allows for higher accuracy in quantitative analyses [15], therefore the single-copy histone-H3 gene was selected for the preliminary endpoint PCR assay. The primers (QEH3-F and QEH3-R) were designed against a portion of the histone-H3 ORF identified in the draft genome of *Q. eucalypti* isolate CBS 118844. The newly developed assay was shown to be specific for *Q. eucalypti* by testing a panel of 30 *Q. eucalypti* isolates and 19 non-target species associated with *Eucalyptus* spp.

High-sensitivity detection methods depend on the pathogen's abundance in the plant, particularly in cases of asymptomatic latent infections. This assay was able to detect the pathogen in both symptomatic and asymptomatic infected eucalyptus plants. The use of the histone H3 region ensured high analytical sensitivity, allowing the detection of *Q. eucalypti* DNA concentrations in symptomatic plants ranging from approximately 0.3 to 1.6 pg, depending on the disease severity. While, in asymptomatic plants the pathogen can be detected at levels as low as 10 fg DNA, which is the limit of detection for the assay. Additionally, the pathogen was also detected when asymptomatic infected eucalyptus leaf and stem tissues were mixed with healthy plant material in a ratio of 1:10, demonstrating the high sensitivity of the assay. To enable the simultaneous detection of pathogen and plant internal control, the cPCR assay was duplexed with an assay targeting the plant *CDK8* gene [25]. Although duplexing or multiplexing can compromise the sensitivity of pathogen-specific assays [33], the duplexed cPCR assay was able to detect both *Q. eucalypti* and the plant internal control in a single reaction without any interference or reduction in sensitivity. Furthermore, the robustness and effectiveness of the test in other laboratories was demonstrated by comparable results obtained in interlaboratory tests.

5. Conclusions

In conclusion, this newly developed real-time PCR assay can be used for conducting more detailed epidemiological studies, testing plant material in known *Q. eucalypti* distribution areas for early management practices, or gathering information for resistance breeding programs. In addition, this SYBR Green-based qPCR assay also offers cost advantages, being much less expensive than other diagnostic tools such as qPCR using fluorescent probe chemistry or real-time lamp.

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