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Enhanced TaqMan qPCR Assay for *Phytophthora cinnamomi* Detection and Quantification

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ABSTRACT

Phytophthora cinnamomi is one of the world's most invasive plant pathogens, requiring accurate detection in both plant and soil samples for effective disease management. This study compares existing qPCR assays with newly developed assays targeting the internal transcribed spacer (ITS) gene for improved detection. Our findings revealed that few current primers reliably distinguish *P. cinnamomi* from closely related species, and even those that did showed inadequate sensitivity. To address this, we designed novel primers and a species-specific probe targeting the ITS region, achieving consistent amplification with a detection limit of 10 fg. The new assay successfully detected *P. cinnamomi* in both artificial and symptomatic samples, ensuring rapid and precise identification. This optimised qPCR assay detects and quantifies *P. cinnamomi* in soil, providing superior sensitivity and specificity over existing methods and supporting more accurate pathogen management.

1 | Introduction

Alien Invasive Forest Phytophthoras (AIFPs), particularly *Phytophthora cinnamomi* Rands (1922), pose a severe threat to the biodiversity and health of chestnut (*Castanea sativa* Mill.) and evergreen oak ecosystems in Southern Europe and the Mediterranean (Vannini et al. 2021; Morales-Rodríguez et al. 2025). *P. cinnamomi* Rands (1922) is responsible for root rot and stem cankers, leading to widespread tree mortality and significant economic losses in agriculture, forestry and horticulture (Hardham and Blackman 2018; Colavolpe et al. 2023). The current ink disease epidemic, driven by *P. cinnamomi* and other *Phytophthora* species, is devastating large areas of *C. sativa* Mill., *Quercus suber* L. and *Q. ilex* L. forests, causing symptoms like crown dieback, leaf chlorosis and root necrosis (Vannini and Morales-Rodriguez 2022; Telfer et al. 2015). *P. cinnamomi* is one of the most frequent species present in Mediterranean nurseries and plantings (Jung et al. 2016) which has contributed to the pathogen's spread into new areas. Initially prevalent in tropical and subtropical regions, *P. cinnamomi* is now expanding its range into cooler, central regions of Europe, such as Germany, driven by global warming and rising temperatures (Nechwatal and Jung 2021). Experimental studies have also demonstrated that *P. cinnamomi* exhibits significant phenotypic plasticity at low temperatures (Khaliq et al. 2020).

The management of ink disease in forests and orchards relies on both preventive and curative strategies that need to be applied on a regional scale to be effective (Vannini and Morales-Rodriguez 2019). Therefore, an efficient monitoring system for the presence and early detection is needed. Accurate and rapid detection plays a fundamental role in the management of *P. cinnamomi*. Moreover, the possibility of monitoring the distribution and accumulation of the pathogen as a function of host phenology and environmental parameters is a paramount requirement in clarifying the pathobiome (Turco et al. 2021). Traditional methods for detecting *P. cinnamomi* include sample isolation from symptomatic plant tissues and baiting from soil (Erwin 1996). However,

these methods are time-consuming and are often hampered by the low abundance of the pathogen in soil and the presence of microbial competitors (Hardham 2005). Given the limitations of these traditional methods, molecular diagnostic tools have emerged including conventional polymerase chain reaction (PCR) (Schena et al. 2008), nested PCR (Langrell et al. 2011), loop-mediated isothermal amplification (LAMP), Recombinase Polymerase Amplification (RPA) (Dai et al. 2019, 2020) and real-time PCR (Engelbrecht et al. 2013; Kunadiya et al. 2019). Among these, quantitative PCR (qPCR) is widely recognised for its high sensitivity and accuracy in detecting and quantifying pathogens. However, the effectiveness of molecular diagnostic tools is highly dependent on their specificity and sensitivity in identifying pathogens in diverse environmental samples (Kunadiya et al. 2017). Although PCR is sensitive and robust, it has some limitations. The assay specificity depends on the primers used and its sensitivity makes it susceptible to false positives if care is not taken to avoid contamination (Kunadiya 2018).

The identification of *Phytophthora* species is mainly based on the sequencing of the Internal Transcribed Spacer (*ITS*) region (Cooke et al. 2000; Seifert 2009; Grünwald et al. 2011). The *ITS* region is considered the best available genetic marker to identify *Phytophthora* at the clade level, as almost all known *Phytophthora* taxa have been sequenced for the *ITS* region (Yang and Hong 2018).

Existing real-time methods for detecting *P. cinnamomi* often rely on multi-copy mitochondrial genes, such as the *atp9-nad9* loci (Bilodeau et al. 2014; Miles et al. 2017) and *Cox2* (Kunadiya et al. 2019). While these mitochondrial targets offer some advantages, high-copy number regions such as the *ITS* and the elicitin gene family generally provide greater sensitivity, particularly in detecting low pathogen loads, compared to single-copy genes such as *Ypt1* and *β-tubulin* (Martin 2013).

In this study, we aimed to develop a new qPCR assay targeting the *ITS* region and evaluate its performance in the detection and quantification of *P. cinnamomi* compared to two of the published assays (Kunadiya et al. 2019; Haegi et al. 2024). Indeed, we aimed to evaluate which of these assays has the best sensitivity when applied to environmental samples and provides effective quantification, a crucial parameter for estimating disease risk potential and for guiding control strategies to limit the spread of *P. cinnamomi*.

2 | Material and Methods

2.1 | Quantitative PCR Primers and Probe Design

The internal transcribed spacer (*ITS*) region was chosen as the target for our detection assay, due to its widespread use as a molecular barcode for identifying *Phytophthora* species. To design qPCR primers specific to *P. cinnamomi*, sequences from various *Phytophthora* species were retrieved from the GenBank database. These sequences were aligned using MEGA 10 software (Kumar et al. 2018), and the alignment was meticulously examined to identify variations that could serve as target regions for primer design. Once the target region was identified, it was analysed using BLASTn, and sequences with high similarity to the target region were downloaded and aligned, allowing us to prioritise these species for further analysis. Primer design was then conducted using Primer3 (Rozen and Skaletsky 1999) with default search parameter criteria to accurately identify effective and compatible primer sequences. The analytical specificity of primer pairs and probes was preliminarily tested in silico using Primer-BLAST (Ye et al. 2012). Selected primers were synthesised and HPLC purified by Eurofins Genomics (Eurofins Genomics GmbH, Konstanz, Germany).

We also tested two alternative protocols. The first, by Kunadiya et al. (2019), uses species-specific primers and the PCIN probe, targeting a mitochondrial locus encoding subunit 2 of cytochrome c oxidase (*Cox2*). The second assay, recently developed by Haegi et al. (2024), targets the nuclear single-copy *Ypt1* gene that encodes a ras-related protein. All the primers and probes tested in this study are listed in Table 1. The three assays were evaluated for sensitivity and efficiency, allowing a comparative analysis of their performance.

TABLE 1 | Primers and probes used in this study.

Name	Target gene	Direction	Sequence	Reference
PhygenF	*ITS*	Forward	CACGTGAACCGTATCAACCC	This study
PhygenR	*ITS*	Reverse	CCCCGAGAGGGACCCAAA	
ITSCIN probe[a]	*ITS*		CTGCTCTGGGCGGCG	
PCIN147F2	*Cox2*	Forward	CCAGCAACTGTTGTGCATGGA	Kunadiya (2018)
PCIN246R2	*Cox2*	Reverse	ATAATAAAGCAAATGATGGT	
PCIN probe[b]	*Cox2*		TGAAATTATTTGGACTTCTATACCTGC	
P. cinn 3.4F	*Ypt1*	Forward	TTTGTGAGTGCCGAGACAAG	Haegi et al. (2024)
P. cinn 3.78R	*Ypt1*	Reverse	GCACAGAAACAACAACGACG	
P. cinn 3.3 probe[c]	*Ypt1*		CCTGTCTGCCCCATTCAACAGA	

[a]6-carboxyfluorescein (FAM) dye at the 5′ end with the Black Hole Quencher1 (BHQ1) at the 3′ end.
[b]Double-quencher probes 5′6-carboxyfluorescein, ZEN internal quencher, Iowa Black fluorescent quencher (5 6-FAM/ZEN/3 IBFQ).
[c]6-carboxyfluorescein (FAM) dye at the 5′ end with the Black Hole Quencher1 (BHQ1) at the 3′ end.

2.2 | Quantitative PCR (qPCR) Conditions

Quantitative PCR (qPCR) reactions contained 5 μL of 2X GoTaq Probe qPCR Master Mix (PROMEGA), 300 nM of both forward and reverse primers, 100 nM of TaqMan probe, 1 μL of template DNA and ultrapure water to a final volume of 10 μL per reaction. All the amplifications were performed in a RotorGeneQ (Qiagen, Hilden). The thermic amplification profile for the *ITS* gene started with an initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 64°C for 15 s and 72°C for 20 s. Fluorescence was recorded at the end of each extension step, and Cq values were automatically determined by the instrument. Each sample was run in duplicate to ensure repeatability.

2.3 | Assay Specificity

The specificity of the designed primers and TaqMan probe was initially assessed through in silico screening using Primer-BLAST, whereby the NCBI DNA sequence database was queried to identify potential matching sequences in unrelated microorganisms. Additionally, we assessed the in silico specificity of the primers against *Phytophthora* species commonly co-occurring with *P. cinnamomi*, such as *P. quercina* and *P. castanetorum*.

Subsequently, the analytical specificity of the qPCR assay was evaluated using genomic DNAs derived from 17 *Phytophthora* spp. and *Pythium* spp. isolates, as detailed in Table 2. The DNA samples were obtained from in-house collections curated by the Department for Innovation in Biological, Agro-Food and Forest Systems at Tuscia University. The DNA concentrations had been previously quantified using either an OPTIZEN NanoQ Lite (Keen Innovation Technologies) or a Qubit dsDNA assay kit (Thermo Fisher Scientific, Waltham, USA), and were subsequently diluted to a uniform concentration of 5 ng/μL for the specificity assay. The DNA of a known strain of *P. cinnamomi* was used as a positive control, while PCR-grade water was used as a negative control. A qPCR reaction was considered positive if an amplification curve with a Cq value below the limit of detection was consistently observed in all replicates, as detailed in the results section.

2.4 | Construction of Standard Curves for Quantitative Analyses

The sensitivity of all three assays targeting the *ITS* gene, *Cox2* and *Ypt1*, respectively, was tested using a 10-fold serial dilution of *P. cinnamomi* strain L313.

To construct the curve, genomic DNA was serially diluted in sterile water to create seven final concentrations, ranging from 10 ng/reaction to 1 fg/reaction. Each concentration was amplified in triplicate to ensure accuracy. Negative control reactions were performed, replacing template DNA with water.

For *Cox2*, amplifications were performed with an initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 64°C for 15 s and 72°C for 10 s, with fluorescence recorded at the end of each extension phase.

TABLE 2 | List of oomycete species tested in our study.

	Genus and species	Isolate code	Accession number	qPCR result
1	*Phytophthora cactorum*	P40	PQ569739	–
2	*Phytophthora xcambivora*	P105	PQ569740	–
3	*Phytophthora citrophthora*	P6	PQ569741	–
4	*Phytophthora cryptogea*	P84	PQ569742	–
5	*Phytophthora multivora*	P440	PQ569743	–
6	*Phytophthora nicotianae*	A004	PQ569744	–
7	*Phytophthora gonapodyides*	P55	PQ569745	–
8	*Phytophthora lateralis*	P243	PQ569746	–
9	*Phytophthora cinnamomi*	P51	PQ569747	+
10	*Phytophthora cinnamomi*	P123	PQ569748	+
11	*Phytophthora cinnamomi*	DM51	PQ569759	+
12	*Phytophthora cinnamomi*	L313	PQ569750	+
13	*Elongisporangium helicandrum*	DM20	PQ569751	–
14	*Elongisporangium undulatum*	DM22	PQ569752	–
15	*Phytopythium vexans*	DM45	PQ569753	–
16	*Pythium pachycaule*	DM40	PQ569754	–
17	*Elongisporangium anandrum*	DM13	PQ569755	–

For *Ypt1*, amplifications involved an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 20 s and 62°C for 20 s. Fluorescence was measured at the annealing–extension phase at 62°C during each PCR cycle.

The standard curve was generated for each gene by plotting the DNA concentrations against the corresponding Cq values. The coefficient of determination (R^2) was calculated to assess the linearity of the curve, while the amplification efficiency (E) was derived from the slope, indicating the relationship between Cq values and the amount of target DNA. The performance of the qPCR assay was evaluated following the criteria established by

Broeders et al. 2014, which specify an efficiency between 90% and 110%, linearity ($R^2 \geq 0.98$) and repeatability (relative standard deviation $\leq 25\%$).

2.5 | Validation and Comparison of the Three Assays in a Mesocosm Experiment

2.5.1 | Mesocosm Experiment

Seedling plants (10 leaves stage) obtained from chestnut seeds sterilised and sown in plastic root trainers, with 1 acorn per cell, were used. Inoculum was prepared according to the methodology reported by Morales-Rodriguez et al. (2016); *P. cinnamomi* isolates were grown in flasks containing sterile millet (*Panicum miliaceum*) for 3 weeks at 20°C. Colonised millet grains were mixed with the potting mix at a concentration of 3% (vol/vol) in 1 L pot per seedling. The potting mix contained autoclaved peat (Vigorplant, Italy) and vermiculite (4:1 vol/vol). The control seedlings received only sterile millet grains. The overall experiment included 10 inoculated and 10 control seedlings.

2.5.2 | DNA Extraction From Soil

For soil DNA extraction from pot plants, 400 mg of soil was processed using the Macherey-Nagel NucleoSpin Soil Kit according to the manufacturer's instructions. The sample, placed in the NucleoSpin Bead tubes with ceramic beads, was homogenised with the TissueLyser II (Qiagen, Germany) for 5 min at 25 Hz.

After the extraction procedures, the concentration and quality of DNA were checked using Qubit with the dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) and the OPTIZEN NanoQ Lite (Keen Innovation Technologies), DNA was stored at −20°C until use.

2.5.3 | Detection of *P. cinnamomi*

Detection of *P. cinnamomi* in soil samples was performed using the newly developed *ITS* assay and compared to assays targeting the *Cox2* and *Ypt1* genes.

For both *Cox2* and *Ypt1*, a nested qPCR reaction was required, following the protocols described by Kunadiya et al. (2019) and Haegi et al. (2024).

Each sample was analysed in duplicate, and the final value represented the average of the two technical replicates. The quantity of *P. cinnamomi* DNA was extrapolated from the standard curves based on Cq values and normalised as outlined above.

2.6 | Data Analysis

The threshold value to distinguish positive or negative qPCR results was automatically defined, and the standard curves were generated by the Rotor-Gene Q software (version 2.1.0.9).

Linear regressions of the qPCR standard curves were also calculated by this software. The amplification efficiency was estimated from the slopes of the standard curves using the equation

$$E = 10^{-1/\text{slope}} - 1$$

For absolute quantification, the standard curve was used to estimate the target DNA concentrations in the analysed samples. Data analysis was performed in Microsoft Excel, where the average Cq, standard deviation and coefficient of variation were calculated for each sample.

3 | Results

3.1 | Primers and Probe Design

The primers and probe were designed specifically targeting the *ITS* region (Figure 1; Table 1). A total of 32 gene sequences, representing 14 different *Phytophthora* species, were screened for in silico specificity (Figure 1). The *ITS* region amplified by the primers Phygen-F and Phygen-R, along with the specific probe ITSCIN (Figure 1), exhibited interspecific variability, enabling clear differentiation of *P. cinnamomi* from the other species studied. The resulting amplicon had a length of 126 bp.

3.2 | qPCR Primer Specificity

The preliminary in silico assessment of the specificity of the primers was achieved through Primer-Blast analysis, exploring the NCBI DNA sequence database and excluding the presence of matching sequences in other microorganisms.

In the in silico analysis to evaluate the specificity of our primers and probe against *P. quercina* and *P. castanetorum*, we identified nine SNPs in the probe and three in the reverse primer. Additionally, gaps were observed at the end of the reverse primer, where these species failed to align with *P. cinnamomi*.

Following that, 13 DNA samples were tested, including eight samples from other *Phytophthora* species, distinct from *P. cinnamomi*. The assay successfully amplified all *P. cinnamomi* samples with high reliability, indicating that it is effective in targeting the DNA of this species. In contrast, the non-target DNA samples from the other *Phytophthora* species yielded negative results, as no amplification occurred (Table 2). This demonstrates that the assay is highly specific to *P. cinnamomi*, with no cross-reactivity observed with other closely related species.

3.3 | Comparison of qPCR Assay Sensitivities on *P. cinnamomi*

The sensitivity of three qPCR assays was compared, each targeting a different gene: *Cox2, Ypt1 and ITS*, respectively. For the *Cox2* assay developed by Kunadiya et al. (2019), standard curves were generated by plotting seven 10-fold serial dilutions of DNA from the pure fungal culture of strain L313b against the cycle threshold (Ct) values of qPCR replicates. The resulting curve was linear, with a determination coefficient (R^2) of 0.99 and a

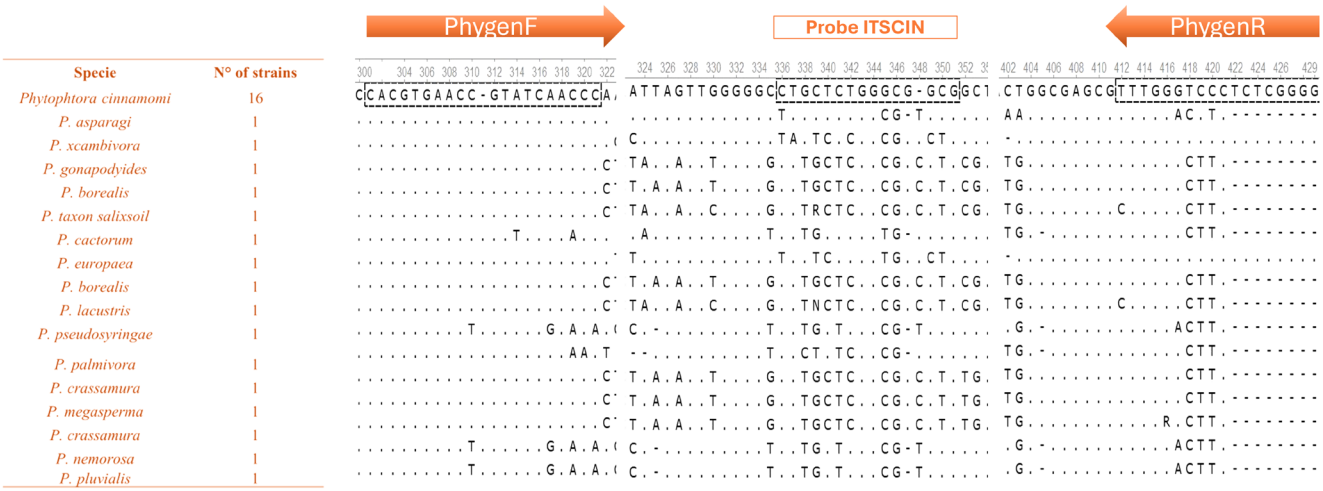

FIGURE 1 | Alignment of primers designed on the ITS gene of *Phytophthora cinnamomi* and the closest available sequences of species of the genus. The mismatching nucleotides of primers to the sequences of *P. cinnamomi* (first line) are reported. The numbers above the alignment refer to positions in *P. cinnamomi* strains. The accession numbers of the sequences from NCBI Database are reported in Table S1.

reaction efficiency of 106% (Figure 2A,B). In terms of sensitivity, the lowest DNA concentration yielding positive amplification was 100 fg.

Using the same 10-fold DNA dilutions, the assay with *Ypt1* primers and probe, developed by Haegi et al. (2024), detected a minimum pathogen DNA concentration of 1 pg, which is 10 times higher than the *Cox2* assay. The reaction efficiency for this assay was 91%, with an R^2 of 0.99 (Figure 2C,D).

In the assay employing our newly designed primers targeting the *ITS* gene, the lowest detectable pathogen DNA concentration was 10 fg, which is 10 times more sensitive than the *Cox2* assay and 100 times more sensitive than the *Ypt1* assay. The reaction efficiency was 93%, with an R^2 of 0.99 (Figure 2E,F).

3.4 | Validation and Comparison of the Three Assays in a Mesocosm Experiment

In this mesocosm experiment, the qPCR assays targeted *Cox2*, *Ypt1* and *ITS* genes to detect the presence of the pathogen *P. cinnamomi* in soil samples. The experiment aimed to evaluate the efficiency of these assays in identifying the pathogen in infested and non-infested samples. The *ITS* assay successfully detected *P. cinnamomi* in 10 out of 10 infested soil samples, with Ct values ranging from 24.05 to 34.25 (Table 3). This demonstrates the assay's consistency and reliability in detecting the pathogen across a broad range of concentrations.

In contrast, the *Cox2* and *Ypt1* assays were less efficient. The *Cox2* assay detected the pathogen in nine out of 10 samples but required nested qPCR for soil samples to enhance sensitivity. The *Ypt1* assay was the least effective, detecting the pathogen in only 6 out of 10 samples, suggesting it may not be reliable for monitoring the pathogen in soil environments. Notably, none of the control seedlings (S1–S5, S11–S15) showed any positive amplification for any of the three assays, confirming their specificity for *P. cinnamomi*. The absence of amplification in these controls further validates the correlation between positive results and the presence of the pathogen in infested samples.

4 | Discussion

The detection of *P. cinnamomi* has long posed challenges due to its soil-borne nature and wide host range. Traditional diagnostic techniques, such as isolation on selective media and morphological identification, are time-consuming and often limited by the pathogen's low abundance in the soil and the presence of microbial competitors (Hardham 2005). In this study, our newly developed qPCR assay targeting the *ITS* gene demonstrates enhanced specificity and sensitivity compared to existing methods. Several studies have extensively documented the use of qPCR for detecting *Phytophthora* species in environmental samples, underscoring its value in pathogen monitoring. Common molecular targets include the *ITS* region and the *Cox2* gene, both of which are highly conserved across *Phytophthora* species, making them suitable for designing species-specific primers (Martin 2013; Riddell et al. 2019). *ITS* gene has gained prominence due to its ability to discriminate between closely related species (Cooke et al. 2000), though its sensitivity may vary depending on the environmental context and sample matrix (Scibetta et al. 2012). Additionally, the *Ypt1* gene has been used for *Phytophthora* detection (Haegi et al. 2024); however, despite its usefulness, *Ypt1* often shows lower detection efficiency in complex environmental samples compared to *ITS*-based assay. In our study, the *ITS* assay's broad Cq range (24.05–34.25) reflects its ability to detect varying concentrations of the pathogen, from low-level infestations to more extensive colonisation. This wide range may explain its high detection rate, even in soil, which often presents challenges due to organic matter and competing microbes. Furthermore, the specificity of the *ITS* assay, as demonstrated by the lack of amplification in non-infested samples, aligns with the literature's consensus on its robustness for environmental pathogen monitoring.

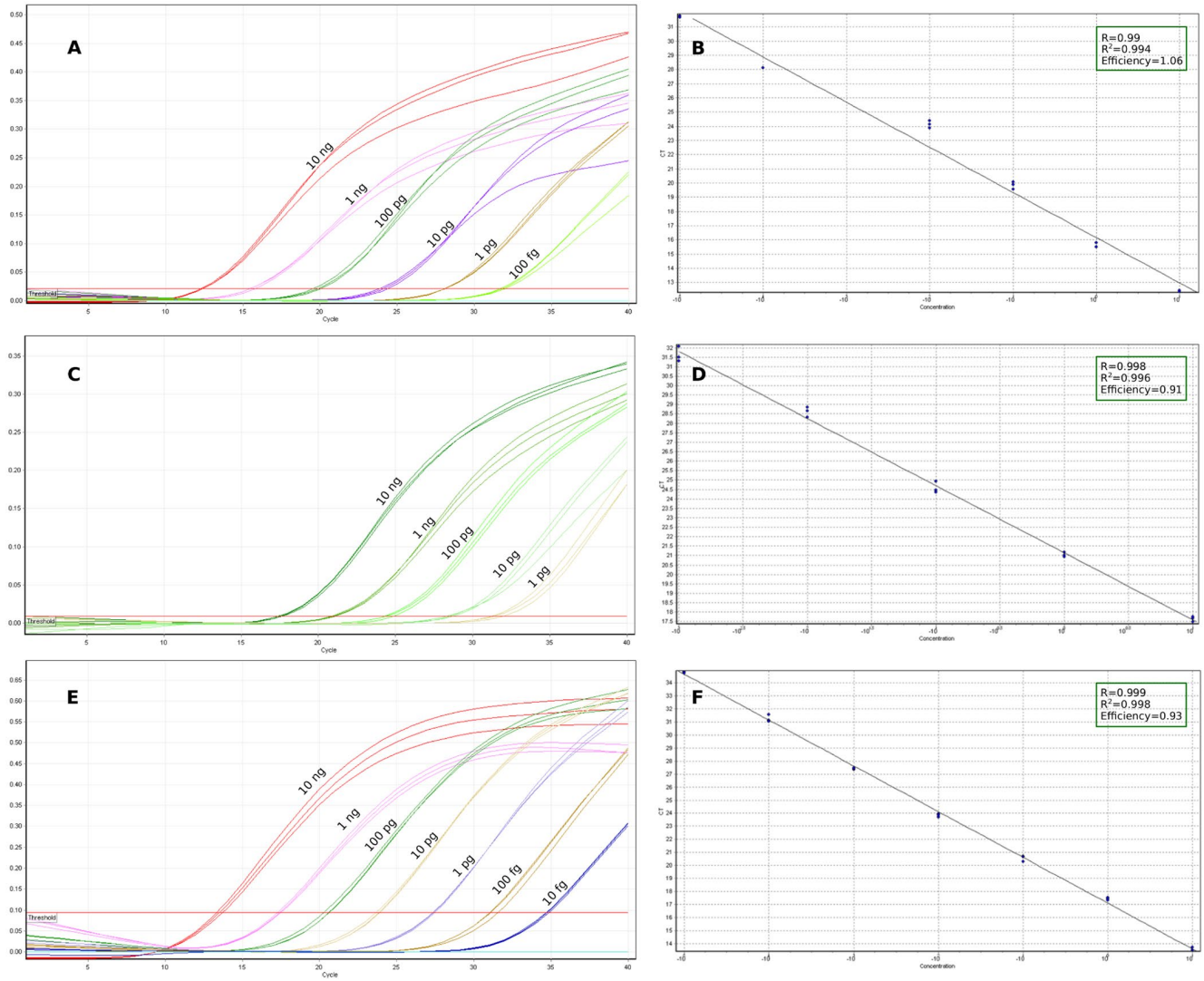

FIGURE 2 | (A) Amplification curves of the qPCR sensitivity test using *Cox2* primers and probe using 10-fold serial dilutions of *Phytophthora cinnamomi* pure DNA ranging from 10 ng/rxn to 1 fg/rxn. (B) Standard curve obtained with 10-fold dilutions using *Cox2* primers and probe of pure DNA of *Phytophthora cinnamomi* and related statistics. (C) Amplification curves of the qPCR sensitivity test using *Ypt1* primers and probe using 10-fold serial dilutions of *Phytophthora cinnamomi* pure DNA ranging from 10 ng/rxn to 1 fg/rxn. (D) Standard curve obtained with 10-fold dilutions using *Ypt1* primers and probe of pure DNA of *Phytophthora cinnamomi* and related statistics. (E) Amplification curves of the qPCR sensitivity test using *ITS* primers and probe (designed in this study) using 10-fold serial dilutions of *Phytophthora cinnamomi* pure DNA ranging from 10 ng/rxn to 1 fg/rxn. (F) Standard curve obtained with 10-fold dilutions using *ITS* primers and probe of pure DNA of *Phytophthora cinnamomi* and related statistics.

The *ITS* region is recognised for its utility in oomycete identification due to its interspecies variability and the presence of conserved flanking regions ideal for primer binding (Cooke et al. 2000). Our results align with previous studies that highlight *ITS* as a reliable target for detecting *P. cinnamomi*. For instance, Langrell et al. (2011) achieved similar success using *ITS*-based assays to detect *P. cinnamomi* in chestnut grove soils.

While the *Cox2* gene is frequently targeted in qPCR, its detection efficacy can be limited by lower copy numbers in environmental samples. Our findings reflect this limitation, with the *Cox2* assay detecting 9 out of 10 samples but requiring nested qPCR for soil samples. The need for nested amplification introduces an infested additional step, increasing the assay's complexity and potential for contamination (Lau and Botella 2017).

When we tested the sensitivity of the *Ypt1* assay developed by Haegi et al. 2024, the qPCR method demonstrated a detection limit of 1 pg, while Haegi et al. (2024) reported a lower detection threshold of 50 fg of total genomic DNA. This difference can be attributed to the use of different qPCR instruments and reagents used during the experiments. Furthermore, the *Ypt1* assay underperformed during our validation on the mesocosm experiment, detecting the pathogen in only 6 out of 10 samples, even with the nested qPCR. The higher Cq values observed for *Ypt1* suggest either lower target abundance or diminished amplification efficiency, consistent with earlier findings by Schena et al. (2006).

The variability in the assay's performance is likely tied to factors such as soil composition, pathogen load and environmental

TABLE 3 | Results of the validation and comparison of the three assays in the mesocosm experiment.

Sample code	Presence of symptoms	*Cox2* Cq value (± SD)	*Ypt1* Cq value (± SD)	*ITS* Cq value (± SD)
S1	No	—	—	—
S2	No	—	—	—
S3	No	—	—	—
S4	No	—	—	—
S5	No	—	—	—
S6	Yes	21.37 (±0.05)	—	24.05 (±0.2)
S7	Yes	24.69 (±0.1)	35.04 (±0.1)	26.965 (±0.12)
S8	Yes	27.27 (±0.02)	—	32.035 (±0.03)
S9	Yes	21.48 (±0.04)	34.63 (±0.05)	25.04 (±0.04)
S10	Yes	19.9 (±0.08)	—	25.325 (±0.09)
S11	No	—	—	—
S12	No	—	—	—
S13	No	—	—	—
S14	No	—	—	—
S15	No	—	—	—
S16	Yes	23.37 (±0.1)	35.53 (±0.2)	29.3 (±0.19)
S17	Yes	28.48 (±0.06)	35.73 (±0.15)	32.55 (±0.19)
S18	Yes	28.14 (±0.03)	35.97 (±0.3)	33.755 (±0.38)
S19	Yes	31.9 (±0.15)	34.63 (±0.2)	33.555 (±0.62)
S20	Yes	—	—	34.25 (±0.43)

stress, which can influence *Ypt1* expression. The *Ypt1* assay's higher Cq values and lower detection frequency make it less reliable for monitoring *P. cinnamomi* in environments where pathogen concentration is low. On the other hand, soil DNA extraction remains problematic due to the diverse physical and chemical properties of soils and their microbiome complexity. Even though Haegi et al. (2024) improved extraction by analysing larger soil samples (5–15 g), they also suggested that further increases in the starting soil material, up to 20–100 g, could enhance DNA yield. However, this approach presents significant economic and logistical challenges, making it less feasible for routine environmental monitoring applications. Thus, both the assay's performance and the limitations in soil DNA extraction highlight the complexities in effectively monitoring *P. cinnamomi* in environmental samples.

Conversely, our *ITS* assay specific to *P. cinnamomi* achieved a detection limit of 10 fg of total genomic DNA, comparable to other real-time diagnostic tools for *Phytophthora* species (Bilodeau et al. 2014; Schena et al. 2006; Verdecchia et al. 2021). Schena et al. (2006) reported a sensitivity limit of 100 fg using the *Ypt1* gene target for qPCR across various *Phytophthora* species. Bilodeau et al. (2014) developed qPCR methods targeting the atp9-nad9 mitochondrial locus for 13 *Phytophthora* species, including the *P. citricola* species complex, with a sensitivity limit of < 100 fg. Similarly, Verdecchia et al. (2021) demonstrated that *P. cactorum* DNA could be detected down to 10 fg using a qPCR *ITS*-based assay, while the *Ypt1*-based assay detection limit was 1 pg of DNA.

Given the serious economic and ecological damage caused by *P. cinnamomi*, rapid and accurate detection methods are crucial for effective management (Cahill et al. 2008). The results of this study, coupled with supporting literature, suggest that the *ITS* assay is the most reliable and efficient tool for monitoring *P. cinnamomi* in soil. Its sensitivity, even without the need for nested PCR, and its ability to detect the pathogen across a wide range of concentrations make it well suited for early detection and management efforts in agriculture and natural ecosystems.

In contrast, while the *Cox2* assay can detect *P. cinnamomi*, its reliance on nested qPCR limits its practicality for routine diagnostics, particularly in resource-limited settings.

Moving forward, combining molecular diagnostics such as qPCR with next-generation sequencing (NGS) or metagenomics could offer even deeper insights into the environmental presence and spread of *P. cinnamomi* and other soil-borne pathogens (Landa et al. 2021). Indeed, while qPCR targets a specific genetic sequence, metagenomics and NGS have the advantage of detecting a wide range of organisms, including low-abundance *P. cinnamomi* and other co-occurring pathogens. Additionally, these techniques offer valuable insights into overall microbial diversity that go beyond the capabilities of qPCR but remain quite expensive and labor-consuming for routine investigations. Continued optimisation of qPCR assays, for example, through the development of multiplex assays targeting multiple genes simultaneously, holds great potential for improving pathogen detection in complex environments such as soil. Combining single-copy and multi-copy genes for *P. cinnamomi* can enhance both sensitivity and specificity. The single-copy gene ensures precise identification by minimising false positives, while multi-copy genes increase sensitivity, enabling the detection of low pathogen concentrations. This approach could offer a robust and reliable method for detecting *P. cinnamomi* even in the presence of diverse soil microbiota and environmental variability, making it a powerful tool for pathogen monitoring in complex ecosystems.

Author Contributions

M.I.D. performed the laboratory experiments, interpreted the results, prepared the figures and wrote the manuscript in consultation with

A.M., C.M.-R. and A.V., C.M.-R. provided all the isolates and performed the mesocosm experiments. A.M. supervised the project and approved all the analysis. M.I.D., A.M., S.T., C.M.-R. and A.V. discussed the results, provided critical feedback and contributed to the final manuscript.

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Data Availability Statement

Sequence data used and analysed in this research are openly available from Genbank (https://www.ncbi.nlm.nih.gov/genbank/) and the accession numbers are available either in the paper or Supporting Information. Other data supporting the findings of this study are provided in the manuscript and available from the corresponding author upon request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.