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Development and validation of a 28S rDNA-based TaqMan real-time PCR assay for sensitive detection of *Rhizoctonia theobromae* in cassava tissues

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ABSTRACT

Cassava witches' broom disease (CWBD) is an important disease that causes significant production losses. The disease was initially attributed to phytoplasmas; however, inconsistent detection has raised questions regarding their association with the disease. Recent studies have increasingly associated *Rhizoctonia theobromae* (syn. *Ceratobasidium theobromae*) with CWBD, highlighting the need for a more reliable detection method to support phytosanitary, surveillance, and disease management strategies. This study developed and validated a TaqMan-based real-time PCR assay targeting a species-specific 101-bp region of the 28S rDNA of *R. theobromae*. Primers and probe were designed using Geneious Prime and evaluated for specificity using Primer-BLAST and *in vitro* assays. No cross-reactivity was detected with DNA from cassava-associated *Fusarium, Colletotrichum*, *Cladosporium*, *Lasiodiplodia*, and other fungi isolated from cassava tissues. Analytical sensitivity was evaluated using 10-fold serial dilutions of *R. theobromae* genomic DNA from 1 ng/µL to 0.001 fg/µL. The assay consistently detected 10 fg/µL DNA, establishing an analytical limit of detection. The standard curve showed an amplification efficiency of 99.77%, an R^2 of 0.987, and a slope of −3.33. Application of the assay to symptomatic cassava plants demonstrated detection of *R. theobromae* DNA in leaf petiole/midrib, stem, and root tissues, with stem tissue showing the highest detection frequency (95.2%) at harvest stage. Target DNA was also detected in 5 of 33 asymptomatic leaf petiole/midrib samples (13.2%). These findings demonstrate that the developed real-time PCR assay enables sensitive and specific molecular detection of *R. theobromae* and has potential applications in CWBD surveillance, epidemiological studies, and phytosanitary diagnostics.

Keywords: *Rhizoctonia theobromae*, real-time PCR, cassava witches' broom, pathogen detection

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is one of the most important root crops in tropical and subtropical regions worldwide. It is extensively cultivated as a source of food, livestock feed, starch, and raw material for bioethanol production (Parmar et al., 2017; Scaria et al., 2024). Often considered as a food security crop, cassava is capable of tolerating drought, adaptable to marginal soils, and requires relatively low production inputs, making it appealing for resource-constraint, smallholder farmers. In recent years, global cassava production has increased in response to growing demand for food, industrial starch, and renewable energy, accompanied by the expansion and intensification of cassava production systems (FAO, 2024; Howeler et al., 2013; Otekunrin, 2024). The increasing movement and exchange of cassava planting materials, together with the expansion of production areas,

may consequently facilitate the emergence and dissemination of pathogens associated with this crop (Graziosi et al., 2016).

Among emerging cassava diseases, cassava witches' broom disease (CWBD) has become an increasing concern because of its impact on crop production and dissemination into areas where it is not yet reported or known to occur. The disease is characterized by excessive shoot proliferation, shortened internodes, reduced and distorted leaves, vascular discoloration or streaking, and impaired storage-root development, which may result in substantial yield and quality losses (Graziosi et al., 2016; Pardo et al., 2023). In Southeast Asia, CWBD was first reported in Thailand in 2009, and it has since been reported in other countries such as Cambodia, Laos, Vietnam, and the Philippines and, more recently, in South America, indicating an expanding geographic distribution and increasing phytosanitary significance (Howeler et al., 2013; Oliveira et al., 2026; Pardo et al., 2023). Despite its increasing importance, the epidemiology and etiology of CWBD are not yet properly established, and reliable detection of the causal pathogen remains a major constraint to disease surveillance and epidemiological studies.

Phytoplasmas were initially considered potential causal agents of CWBD because the characteristic symptoms resemble those associated with several phytoplasma-induced diseases. Phytoplasmas have been detected in symptomatic cassava plants using PCR-based assays targeting the 16S rRNA gene (Alvarez et al., 2013; Dolores et al., 2023; Flores et al., 2013), and consequently, early diagnostic investigations focused primarily on phytoplasma detection. However, the association between phytoplasmas and CWBD has not been consistently demonstrated across studies or geographic locations (Landicho et al., 2024; Pardo et al., 2023). Phytoplasmas have also been detected in asymptomatic or symptomless cassava plants (Koinuma et al., 2018), raising questions regarding their role in disease development. Moreover, a causal relationship between phytoplasma infection and CWBD has not been conclusively demonstrated through experimental evidence. These research gaps have led to further investigations on the sensitivity of existing diagnostic protocols and explorations of other potential etiological agents that may have a role in disease development.

Recent metagenomic, phylogenetic, microscopic, and pathogenicity-related investigations have increasingly associated the basidiomycetous fungus *Rhizoctonia theobromae* (syn. *Ceratobasidium theobromae*, *Thanatephorus theobromae*, and *Oncobasidium theobromae*) with CWBD. High-throughput sequencing has repeatedly detected the abundance and consistent association of *R. theobromae* in symptomatic cassava, while fungal isolation, tissue localization, phylogenetic analyses, and transmission or pathogenicity-related investigations have provided additional evidence supporting its association with the disease (Cortaga et al., 2026;

Gil-Ordóñez et al., 2024; Leiva et al., 2023). Nevertheless, establishing a definitive causal relationship remains challenging because of the biological characteristics of the fungus and the complexity of CWBD. In particular, *R. theobromae* is difficult to isolate and maintain in axenic culture as it exhibits relatively slow growth and is unable to propagate after subculturing under laboratory conditions (Leiva et al., 2023; Samuels et al., 2012). These challenges complicate studies on its biology, epidemiology, host colonization, and distribution within infected plants. Consequently, sensitive and reliable molecular methods capable of detecting *R. theobromae* directly from cassava tissues are essential for investigating its association with CWBD.

Molecular diagnostics are particularly important for detecting *R. theobromae* when pathogen abundance is low or if symptoms are not yet apparent. Species-specific conventional PCR primers targeting the internal transcribed spacer (ITS) region have been developed for detection of *R. theobromae* associated with cacao (Samuels et al., 2012), while subsequent PCR-based assays for the pathogen in cassava have targeted the CaMK/CaMKL region (Leiva et al., 2023) and 28S rDNA regions (Landicho et al., 2025). The nuclear ribosomal DNA (rDNA) cistron contains regions with different degrees of sequence conservation and variability that can be exploited for fungal identification and molecular detection. Among these, the ITS region is recognized as the primary DNA barcode for fungi because of its relatively high sequence variability and generally strong discriminatory power at the species level (Schoch et al., 2012). In contrast, the 28S large-subunit (LSU) rDNA is more conserved than ITS and is useful for resolving phylogenetic relationships among fungal taxa, although it generally provides lower resolution among closely related species (Fell et al., 2000; Schoch et al., 2012; Scorzetti et al., 2002). Nevertheless, the 28S region can contain diagnostically informative sequence variation that may be exploited for molecular detection when comparative sequence analysis identifies sufficiently discriminatory sites. The 28S rDNA has been successfully employed as a diagnostic target in molecular assays for plant-associated fungi (Kaishian et al., 2024; Phurbu et al., 2026), supporting its potential as an alternative locus for assay development when suitable target-specific sequence variation is present.

More recently, Silva et al. (2026) developed a species-specific TaqMan-based real-time PCR assay targeting the CaMK/CaMKL locus of *R. theobromae* in cassava. Their assay demonstrated high amplification efficiency and specificity and increased detection relative to conventional PCR, particularly in leaf tissues with low or heterogeneous pathogen loads. This recent development demonstrates the value of probe-based real-time PCR for sensitive detection of *R. theobromae,* while also highlighting the importance of evaluating diagnostic targets that are independent of the CaMK/CaMKL locus. TaqMan probe-based real-time PCR combines sequence-

specific hydrolysis probe detection with real-time monitoring of PCR amplification, providing sensitive and specific pathogen detection while eliminating the need for post-amplification processing and reducing the risk of PCR product carry-over contamination (Mirmajlessi et al., 2015; Okubara et al., 2005; Schaad & Frederick, 2002; Schena et al., 2004). These characteristics make the approach well suited for sensitive, specific, and high-throughput detection of *R. theobromae* in cassava tissues.

Despite recent advances in molecular detection, an important diagnostic gap remains concerning the distribution of *R. theobromae* within cassava plants. Detection reliability may depend not only on assay sensitivity but also on the type and physiological condition of the tissue sampled. Previous investigations have focused predominantly on symptomatic leaf tissues, whereas the relative occurrence and detectability of *R. theobromae* in leaves, stems, and roots, particularly across different stages of plant development and in asymptomatic plants, remain unexplored. This is particularly relevant because localized and uneven pathogen colonization may result in false-negative diagnostic results when sampling is restricted to a single tissue or when pathogen abundance falls below the detection threshold (Gil-Ordóñez et al., 2024). Identifying tissues that consistently yield detectable *R. theobromae* DNA would therefore improve sampling strategies, increase diagnostic reliability, and facilitate surveillance of CWBD in field and quarantine settings.

In this study, we developed and validated a TaqMan-based real-time PCR assay (28S assay) targeting a species-specific region of the 28S rDNA of *R. theobromae*. Specifically, we (i) evaluated the analytical performance of the assay, including analytical sensitivity, and specificity, following the principles of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines; (ii) assessed the applicability of the assay for detecting *R. theobromae* in cassava tissues; and (iii) characterized tissue-specific detection of *R. theobromae* DNA in symptomatic and asymptomatic cassava plants at different stages of crop development to identify suitable tissue sources for routine molecular detection and surveillance of CWBD.

MATERIALS AND METHODS

Sample collection

Cassava plants exhibiting typical witches' broom symptoms were initially sampled from a field in Isabela Province, Philippines. Stem cuttings obtained from symptomatic plants were subsequently established in an enclosed field trial at the National Crop Protection Center, University of the Philippines Los Baños (UPLB), Laguna, Philippines. Sample collection was conducted in coordination with local and national authorities in compliance with existing biosecurity protocols.

Leaf petiole and midrib tissues were collected from symptomatic plants during the vegetative stage (6 months after planting). At harvest (9 months after planting), leaf petiole and midrib, stem, and root tissues were collected from the same plants. Leaf and stem samples were collected according to their relative position within the plant canopy and classified as upper (top), middle, or lower (bottom) positions (Supplementary Figure S1). Because plants varied in height, branching pattern, and foliage development, particularly those exhibiting witches' broom symptoms, not all canopy positions were available for every plant. The number and distribution of samples collected from each canopy position are provided in Supplementary Table S1.

Individual samples were placed in clean, resealable bags, transported to the laboratory under cooled conditions, and stored at −80 °C until DNA extraction.

DNA Extraction

Total genomic DNA was extracted from cassava leaf, petiole and midrib tissues, stem bark shavings, and root tissues using a modified DNeasy® Plant Mini Kit protocol (Qiagen®, Hilden, Germany), based on the methods of Green et al. (1999) and Landicho et al. (2025). Approximately 100 mg of tissue was ground to a fine powder in liquid nitrogen using a sterile mortar and pestle. The powdered tissue was homogenized in 2.5% cetyltrimethylammonium bromide (CTAB) extraction buffer at a ratio of 1 mL buffer per gram of tissue, followed by DNA purification using the modified DNeasy® Plant Mini Kit protocol.

Genomic DNA extracted from previously obtained pure cultures of *Rhizoctonia theobromae* and other fungi co-isolated from symptomatic cassava plants, including *Fusarium* sp., *Lasiodiplodia* sp., *Cladosporium* sp., *Alternaria* sp., *Colletotrichum* sp., and other still unidentified fungal isolates, was used for assay validation. These isolates were obtained and characterized in a previous study (Landicho et al., 2025).

DNA concentration and purity were assessed using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, MA, USA) and the QuantiFluor® dsDNA System with a Quantus™ Fluorometer (Promega, WI, USA). Blank extraction controls were included during DNA extraction, while no-template controls (NTCs) were also included in PCR runs to check for cross-contamination.

Primer and Probe Design

The *R. theobromae*-specific primer–probe set was designed in Geneious Prime® version 2024.0.7 (Biomatters Ltd., Auckland, New Zealand) using the full and partial 28S rDNA sequences from *R. theobromae* isolated from cassava and other related taxa within the *Ceratobasidium* and *Rhizoctonia* genera from GenBank (Supplementary Table S2). Primers were designed to amplify a region of the 28S rDNA, while the hydrolysis probe was positioned within an internal sequence region with similarity between *R. theobromae* isolates but variability with non-*R. theobromae* sequences (Figure 1, Supplementary Table S3). The candidate primer–probe set was evaluated *in silico* using Primer-BLAST against the NCBI nucleotide database, including fungal and cassava host sequences (Supplementary Table S4) (Ye et al., 2012). Since Primer-BLAST only supports forward and reverse primer specificity searches, a separate BLASTn search against the NCBI nucleotide database was used to verify the probe sequence (Altschul et al., 1990). Probe matches were assessed according to percent similarities, query coverage, and sequence match relative to the complete 20-nucleotide probe sequence (Supplementary Table S5).

A primer–probe set targeting a 79-bp region of the plant mitochondrial cytochrome oxidase I (COX1) gene previously used in cassava was used as an endogenous control (Adams et al., 2013; Weller et al., 2000). Hydrolysis probes were labeled with 6-carboxyfluorescein (FAM) at the 5′ end and tetramethylrhodamine (TAMRA) at the 3′ end. All primers and probes were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA) and prepared as 10 µM working stocks.

Testing and validation

Real-time PCR conditions

The detection assay of *R. theobromae* 28S rDNA was performed in a final reaction volume of 10 µL containing 5.0 µL TaqMan™ Universal PCR Master Mix, 0.5 µM each of the Cth28S-F and Cth28S-R primers, 0.25 µM Cth28S-P hydrolysis probe, 2.75 µL nuclease-free water, and 1.0 µL DNA template. The final primer and probe concentrations were 0.5 and 0.25 µM, respectively.

Amplification was performed using an Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The thermal cycling consisted of an initial denaturation at 95 °C for 15 min, followed by 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Fluorescence data were captured after the 60 °C annealing and extension step.

The detection assay of endogenous COX1 was performed independently in a separate 10-µL reaction using 5.0 µL TaqMan™ Universal PCR Master Mix, 0.3 µM each of the COX1 forward and reverse primers, 0.1 µM COX1 hydrolysis probe, 3.3 µL nuclease-free water, and 1.0 µL DNA template. The thermal cycling program consisted of an uracil-N-glycosylase (UNG) treatment at 50 °C for 2 min, followed by an initial denaturation at 95 °C for 10 min and 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Fluorescence was also measured after the 60 °C annealing and extension step.

A temperature-gradient experiment was conducted to determine the optimal annealing temperature for the detection of *R. theobromae* 28S. The assay was evaluated across annealing temperatures of 58–63°C. The temperature-gradient experiment showed no significant differences in R. theobromae DNA amplification, so the optimal primer melting temperature (60°C) was selected for subsequent analysis.

The expected size of the 28S rDNA amplicon generated by the designed primers was verified by agarose gel electrophoresis (Supplementary Figure S2). Real-time PCR amplicons were separated on an agarose gel alongside an appropriate DNA molecular-weight marker and visualized under UV transilluminator Applied Biosystems™ iBright™ Imaging System (Thermo Fisher Scientific, Waltham, MA, USA). The observed amplicon size was compared with the expected size based on the primer sequences and target region.

Analytical sensitivity

The analytical sensitivity of the real-time PCR assay was evaluated using 10-fold serial dilutions of purified genomic DNA from *R. theobromae* and cassava. DNA concentrations were quantified using a Qubit Flex fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Serial dilutions were prepared from an initial concentration of 1 ng μL^{-1} to 0.001 fg μL^{-1} using nuclease-free water.

For concentrations below the quantification range of the fluorometer, nominal concentrations were calculated theoretically from the quantified DNA stock concentration based on the dilution factor. Each dilution was tested in three technical replicates using the *R. theobromae*-specific 28S rDNA detection assay and the cassava COX1 internal control assay.

Cycle threshold (Ct) values obtained from the serial dilution series were used to construct standard curves by plotting Ct values against the logarithm of the DNA concentration. Amplification efficiency (E) was determined from the slope of the standard curve. The standard curve, slope, coefficient of determination (R^2), amplification efficiency, and residual error were automatically plotted and calculated by Applied Biosystems Design and Analysis Software v2.6.0 (© 2021 Thermo Fisher Scientific). Assay performance was evaluated based on linearity, amplification efficiency, and reproducibility across the dilution series, in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2025). The analytical limit of detection (LOD) was defined as the lowest DNA concentration at which all three replicate reactions produced detectable amplification. Dilutions below this concentration with inconsistent detection were not considered as LOD.

Analytical specificity

Analytical specificity was evaluated using genomic DNA from fungal isolates previously recovered from witches' broom-symptomatic cassava plants and identified through sequencing of the internal transcribed spacer (ITS) region. The isolates molecularly identified included *Fusarium* sp., *Lasiodiplodia* sp., *Cladosporium* sp., *Alternaria* sp., *Colletotrichum* sp., while additional unidentified fungal isolates associated with cassava tissues were also included in the analysis (see Table 2).

Genomic DNA from a confirmed *R. theobromae* isolate was included as the positive control. Each DNA sample was tested in three technical replicates together with positive and no-template controls. Analytical specificity was evaluated based on amplification of the target 28S rDNA in *R. theobromae* and the absence of detectable amplification in non-target fungal isolates. Since pure cultures of other *Ceratobasidium* species were not available for experimental cross-reactivity testing, closely related taxa were additionally assessed *in silico* using Primer-BLAST and BLASTn (Supplementary Table S4 and S5).

Field evaluation of the assay

The field diagnostic applicability of the developed real-time PCR assay was evaluated using DNA extracted from cassava tissues collected from symptomatic and asymptomatic plants. DNA from symptomatic plants sampled during the vegetative stage (6 months after planting) and during the harvesting stage (9 months after planting) were analyzed using the optimized real-time PCR assay. DNA from a confirmed pure culture of *R. theobromae* was also included as a positive control, while DNA from non-target fungi like *Fusarium* sp., no-

template controls, and extraction controls were included as negative controls in each PCR run. A sample was considered positive for *R. theobromae* when the target 28S rDNA assay produced amplification at Ct <40, and the cassava COX1 internal amplification control also produced successful amplification.

Detection frequency was calculated as the proportion of *R. theobromae*-positive samples relative to the total number of samples tested for each tissue type and canopy position. Mean Ct values and standard deviations were calculated from positive samples. For comparison of detection frequencies among tissue types, samples from the upper, middle, and lower canopy positions were pooled within the leaf petiole/midrib, stem, and root tissues.

Statistical analysis

Differences in *R. theobromae* detection frequencies among tissue types were evaluated using Fisher's exact test computed in R version 4.4.2 (R Core Team, Vienna, Austria) through RStudio version 2024.12.1+563. Pairwise comparisons were conducted among leaf petiole/midrib, stem, and root tissues using two-tailed tests. P-values were adjusted for multiple comparisons using the Bonferroni correction.

Statistical significance was set at $P < 0.05$. The analyses were used to evaluate differences in *R. theobromae* detection frequency among tissue types and to identify the most suitable tissue source for molecular detection of the pathogen in cassava.

RESULTS

Symptom observation and sample collection

A total of 17 cassava plants exhibiting typical witches' broom symptoms were identified at both the vegetative stage (6 months after planting) and harvesting stage (9 months after planting). Symptomatic plants exhibited excessive shoot proliferation, shortened internodes, reduced leaf size, and abnormal branching compared with asymptomatic plants. At harvesting, the symptoms further progressed from witches' broom to a more severe dieback and leaf necrosis in the same plants sampled during the vegetative stage (Supplementary Figure S1). Because symptomatic plants varied in canopy structure, branching pattern, and leaf retention, the availability of upper, middle, and lower canopy positions differed among plants, resulting in unequal numbers of samples among canopy categories (Supplementary Table S1).

Primer and probe design

A real-time PCR assay targeting a 28S rDNA region of *R. theobromae* was designed. The assay comprised the Cth28S-F forward primer, Cth28S-R reverse primer, and Cth28S-P hydrolysis probe. The forward and reverse primers had melting temperatures of approximately 60 °C, with GC contents of 50.0% and 55.0%, respectively, whereas the hydrolysis probe had a melting temperature of 65.8 °C. The target region and locations of the primers and probe are shown in Figure 1. The probe-binding sequence was selected from an internal 28S rDNA region showing discriminatory potential among the reference sequences examined. The final assay targeted a 101-bp region, with the forward primer, probe, and reverse primer positioned at nucleotide positions 351–370, 381–400, and 431–450, respectively, of the reference 28S rDNA sequence (Accession No. PP296691).

In silico specificity analysis using Primer-BLAST did not identify predicted amplification products from cassava DNA or the evaluated non-target fungal sequences under the specified primer-pair criteria. The analysis also included closely related sequences deposited under different taxonomic annotations. Several sequences annotated as *Ceratobasidium* sp. in the BLAST database correspond to *Ceratobasidium theobromae* and were therefore treated as target sequences in the present analysis. Likewise, *Thanatephorus theobromae* is a recognized synonym of *Ceratobasidium theobromae* (Supplementary Table S4).

Independent BLASTn analysis of the 20 nucleotide Cth28S-P probe showed complete identity with the target sequences, with 20/20 (100%) identity and 100% query coverage. Additional non-target BLAST hits showed 19/20 (95%) or 18/20 (90%) identities relative to the complete 20 nucleotide probe, corresponding to partial matches rather than full-length probe matches (Supplementary Table S5).

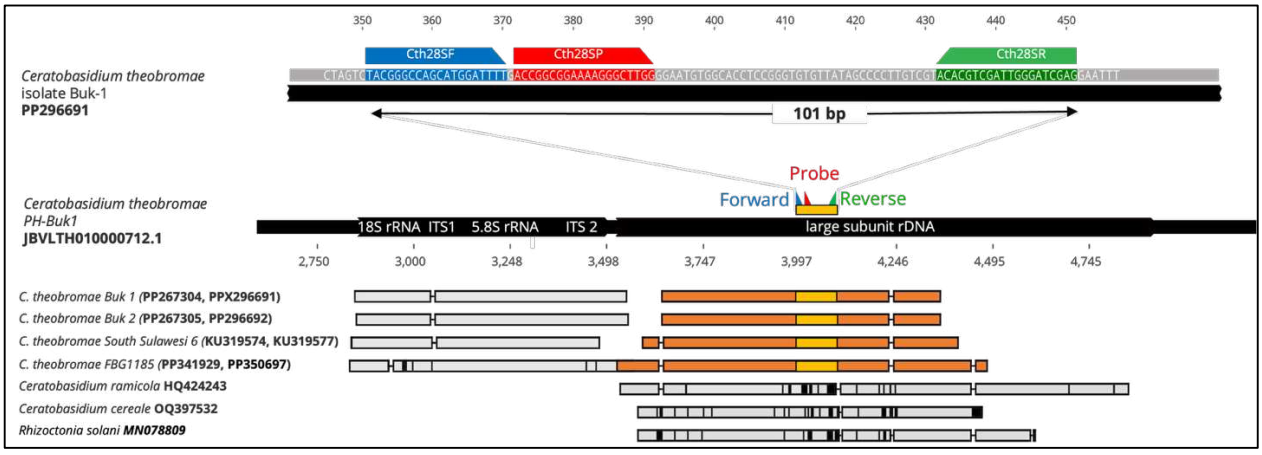


Figure 1. Design of a TaqMan-based real-time PCR assay for *Rhizoctonia theobromae* (28S assay). The figure shows the 28S ribosomal DNA (rDNA) region targeted by the newly designed primers and hydrolysis probe. The forward primer (Cth28SF; blue) spans positions 351–370, the TaqMan probe (Cth28SP; red) spans positions 381–400, and the reverse primer (Cth28SR; green) spans positions 431–450 of *C. theobromae* isolate Buk-1 (Accession No. PP296691). The lower panel shows the relative position of the target region (yellow bands) within the nuclear rDNA and its sequence alignment with representative *C. theobromae* and related *Ceratobasidium* sequences, illustrating the discriminatory region used for assay design.

Optimization and analytical performance of the assay

Temperature-gradient optimization identified 60°C as the optimal annealing/extension temperature for the *R. theobromae* 28S detection assay. Conventional PCR using the designed primer pair produced a single approximately 101-bp amplicon from DNA obtained from *R. theobromae* and symptomatic cassava tissues, whereas no amplification was observed from healthy cassava DNA or no-template controls (Supplementary Figure S2).

The analytical sensitivity of the real-time assay was evaluated using 10-fold serial dilutions of *R. theobromae* genomic DNA ranging from 1 ng μL^{-1} to 0.001 fg μL^{-1} (Table 1; Figure 2). The 28S assay produced a linear standard curve described by $y = -3.33x + 18.93$, with an R^2 of 0.987 and an amplification efficiency of 99.77%. On the other hand, the cassava COX1 endogenous control assay produced a standard curve described by $y = -3.32x + 20.14$, with an R^2 of 0.991 and an amplification efficiency of 100.08%.

For the 28S assay, mean Ct values increased progressively with decreasing template concentration, from 18.44 ± 0.10 Ct at 1 ng μL^{-1} to 36.15 ± 0.47 Ct at 10 fg μL^{-1}. The COX1 assay showed a similar concentration-dependent increase in Ct, from 19.89 ± 0.20 Ct at 1 ng μL^{-1} to 37.07 ± 0.31 Ct at 10 fg μL^{-1} (Table 1). All three replicates were positive at concentrations from 1 ng μL^{-1} through 10 fg μL^{-1} for both assays.

At 1 fg μL^{-1}, amplification became inconsistent, with 2/3 reactions (66.7%) positive for the 28S assay and 1/3 reactions (33.3%) positive for the COX1 assay. No amplification was detected for either assay at 0.1 fg μL^{-1}. A single 28S reaction produced amplification at 0.01 fg μL^{-1} (1/3; 33.3%), but this concentration was not consistently detectable. Therefore, under the experimental conditions used in this study, 10 fg μL^{-1} was determined as the analytical limit of detection (LOD), representing the lowest concentration consistently detected in all three replicate reactions.

The amplification and standard curves showed a consistent concentration-dependent response for both assays (Figure 2). The high R^2 values and amplification efficiencies close to 100% indicated good linearity and amplification performance across the evaluated concentration range.

Table 1. Sensitivity test of real-time PCR protocol for *R. theobromae*

Log_{10} (ng/µL)	Nominal Concentration	28S		COX1	
		% Detection	Ct (Mean ± SD)	% Detection	Ct (Mean ± SD)
0	1 ng/µL	3/3 (100%)	18.44 ± 0.10	3/3 (100%)	19.89 ± 0.20
–1	100 pg/µL	3/3 (100%)	22.15 ± 0.11	3/3 (100%)	23.24 ± 0.30
–2	10 pg/µL	3/3 (100%)	25.55 ± 0.08	3/3 (100%)	26.56 ± 0.49
–3	1 pg/µL	3/3 (100%)	28.98 ± 0.06	3/3 (100%)	30.13 ± 0.53
–4	100 fg/µL	3/3 (100%)	32.81 ± 0.22	3/3 (100%)	33.71 ± 0.44
–5	10 fg/µL	3/3 (100%)	36.15 ± 0.47	3/3 (100%)	37.07 ± 0.31
–6	1 fg/µL	2/3 (66.7%)	37.21 ± 1.51	1/3 (33.3%)	38.15 ± —
–7	0.1 fg/µL	0/3 (0%)	Undetermined	0/3 (0%)	Undetermined
–8	0.01 fg/µL	1/3 (33.3%)	37.84 ± —	0/3 (0%)	Undetermined
–9	0.001 fg/µL	0/3 (0%)	Undetermined	0/3 (0%)	Undetermined

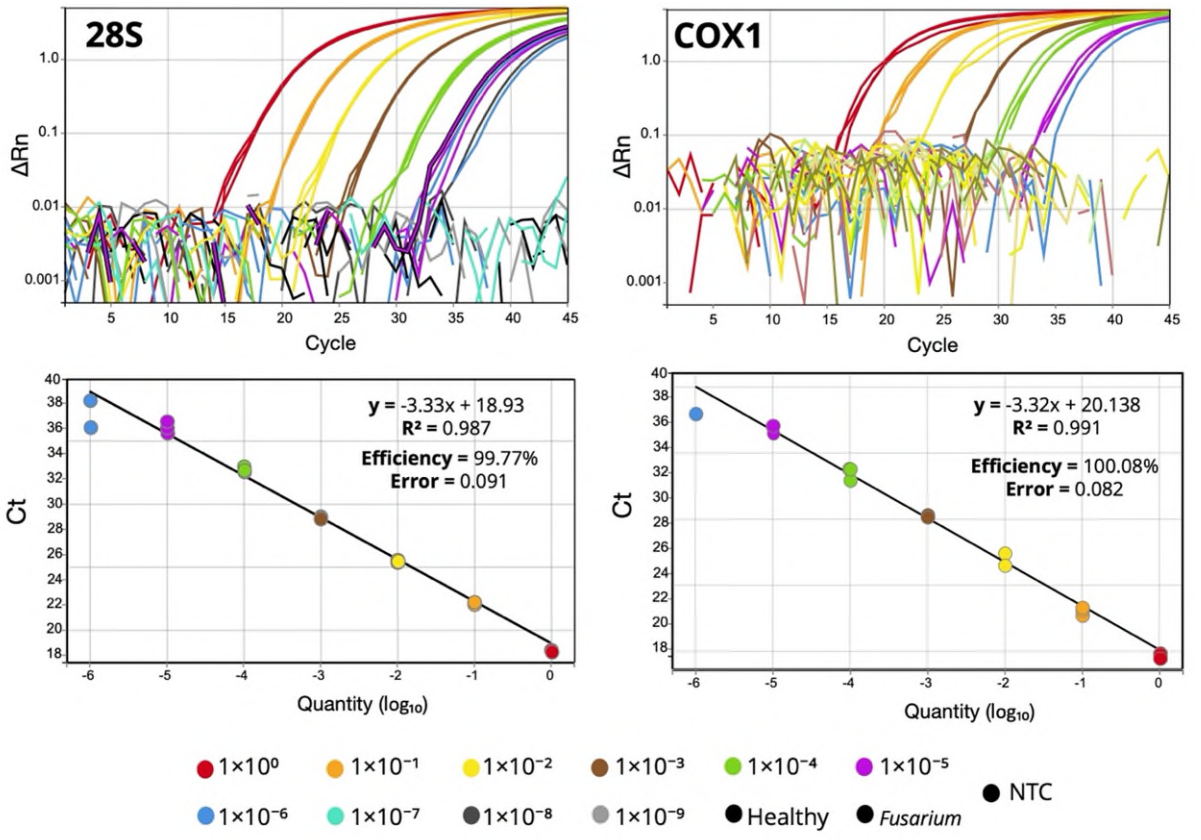


Figure 2. Analytical sensitivity of real-time assays targeting 28S and COX1 genes. The figure displays amplification curves (top panels) and corresponding standard curves (bottom panels) for 10-fold serial dilutions (1×10^{0} to 1×10^{-8}) of target DNA, as well as controls (healthy, *Fusarium*, NTC). The left panel shows data for the 28S gene target, and the right panel shows data for the COX1 gene target. The standard curves include the linear regression equation, R^2 value, efficiency, and error for each assay, demonstrating their linearity, sensitivity, and efficiency.

Analytical specificity

The *R. theobromae* 28S assay showed no detectable amplification of DNA from the non-target fungal isolates tested. No amplification was observed in three replicate reactions containing DNA from *Fusarium* sp., *Lasiodiplodia* sp., *Cladosporium* sp., or *Alternaria* sp. In addition, none of the 12 reactions containing DNA from unidentified fungal isolates associated with cassava produced detectable amplification (Table 2). All non-target reactions remained undetermined, corresponding to 0% detection.

The experimental specificity results were complemented by *in silico* analysis of the primer–probe configuration and independent BLASTn analysis of the hydrolysis probe. Primer-BLAST did not identify predicted amplification products meeting the specified primer-pair criteria among the evaluated non-target sequences. The independent 20 nucleotide probe BLASTn analysis showed 20/20 (100%) identity with the target *C. theobromae* sequences, including sequences deposited in GenBank as *Ceratobasidium* sp. and its synonym, *Thanatephorus theobromae* (Supplementary Tables S4-S5).

In contrast, the remaining non-target BLAST hits showed only partial matches to the complete 20-nucleotide probe, corresponding to 19/20 (95%) or 18/20 (90%) identities (Supplementary Table S5). Thus, the BLASTn results support complete probe sequence conservation among the target sequences examined, while the experimental specificity results demonstrate absence of detectable amplification among the non-target fungal isolates tested.

Table 2. Specificity of the real-time PCR assay against other fungi isolated from cassava.

Other fungi isolated from cassava	Ct mean	No. Positive Detection	Accession[1]
Rhizoctonia theobromae	17.34 ± 0.94	3/3 (100%)	PP267304.1 PP267305.1 PP267306.1 PP267307.1
Fusarium sp.	Undetermined	0/3 (0%)	PV537263.1 PV537264.1
Lasiodiplodia sp.	Undetermined	0/3 (0%)	PV537265.1
Cladosporium sp.	Undetermined	0/3 (0%)	PV537266.1 PV537267.1
Alternaria sp.	Undetermined	0/3 (0%)	PV537268.1 PV537269.1
Other fungus (unidentified)	Undetermined	0/12 (0%)	N/A[2]

[1]ITS sequence used for species identification
[2]No sequence uploaded in the GenBank

Detection of *R. theobromae* in cassava tissues

The validated assay was subsequently applied to cassava tissues collected from symptomatic and asymptomatic plants at different stages of plant development. Detection frequency varied according to crop stage, tissue type, and canopy position (Table 3).

At 6 months after planting, *R. theobromae* DNA was detected at high frequencies in leaf petiole/midrib tissues across all sampled canopy positions. Detection was 83.72% (46/43) in upper canopy leaves, 91.89% (34/37) in middle canopy leaves, and 100% (6/6) in lower canopy leaves. Corresponding mean Ct values were 23.78 ± 8.56, 18.28 ± 3.49, and 20.35 ± 6.90, respectively. The lower-canopy estimate should be interpreted cautiously because of its small sample size (n = 6).

At 9 months after planting, *R. theobromae* DNA was detected in all tissue types examined, although detection frequency varied among tissues and canopy positions. In leaf petiole/midrib tissues, detection was 53.3% (8/15) in the upper canopy, 100% (13/13) in the middle canopy, and 100% (1/1) in the lower canopy. Mean Ct values were 20.08 ± 4.48, 21.06 ± 5.31, and 23.57, respectively. The lower-canopy result was based on a single sample and therefore should not be interpreted as representative of the population. Stem tissues showed consistently high detection frequencies across all canopy positions, with 89.7% (26/29) positive samples in the upper stem, 95.1% (39/41) in the middle stem, and 100% (34/34) in the lower stem. Mean Ct values were 24.40 ± 6.74, 23.76 ± 3.95, and 27.76 ± 4.65, respectively. In root tissues, *R. theobromae* DNA was detected in 81.3% (13/16) of samples, with a mean Ct of 28.50 ± 6.14. Thus, among symptomatic plants at harvest, the assay detected *R. theobromae* DNA in leaf, stem, and root tissues, with the highest overall detection frequency observed in stem tissues.

Among asymptomatic cassava plants, *R. theobromae* DNA was detected in 5 of 33 samples (13.2%), with a mean Ct value of 36.67 ± 1.99. The mean Ct value of the positive asymptomatic samples was substantially higher than those observed in most symptomatic tissues, indicating a lower amount of detectable target DNA. However, the real-time PCR results alone cannot determine whether these positive samples represented latent infection, early infection, low-level colonization, or another biological state.

Table 3. Detection of *R. theobromae* in symptomatic and asymptomatic cassava plants at different crop stages and tissue types.

Crop Stage/ Tissue Type	Location	Ct mean	No. Positive/ Percent Detection
Symptomatic			
6 months			
midrib/petiole	top	23.78 ± 8.56	36/43 (83.72%)*
	middle	18.28 ± 3.49	34/37 (91.89%)*
	bottom	20.35 ± 6.90	6/6 (100.00%)
9 months			
midrib/petiole			
	top	20.08 ± 4.48	8/15 (53.33%)
	middle	21.06 ± 5.31	13/13 (100.00%)
	bottom	23.57 ± -----	1/1 (100.00%)
stem			
	top	24.40 ± 6.74	26/29 (89.66%)
	middle	23.76 ± 3.95	39/41 (95.12%)
	bottom	27.76 ± 4.65	34/34 (100.00%)
root		28.50 ± 6.14	13/16 (81.25%)
Asymptomatic			
midrib/petiole		36.67 ± 1.99	5/33 (13.16%)
*8 samples positive for Cth but negative to COX1			

Tissue-specific detection of *R. theobromae*

For tissue-level comparisons, samples from symptomatic plants at 9 months after planting were grouped into leaf petiole/midrib, stem, and root categories. Overall detection frequencies were 75.9% for leaf petiole/midrib, 95.2% for stem, and 81.3% for root tissues (Figure 3).

Fisher's exact test revealed a significant difference in detection frequency between stem and leaf petiole/midrib tissues (P = 0.0043). Detection was significantly higher in stem tissues than in leaf petiole/midrib tissues. In contrast, the difference between stem and root tissues was not significant (P = 0.0722), and no significant difference was observed between root and leaf petiole/midrib tissues (P = 1.0000) (Figure 3).

Among the tissue categories examined, stem tissue therefore showed the highest overall detection frequency (95.2%) and was significantly more frequently positive than leaf petiole/midrib tissue. Root tissue showed an intermediate detection frequency (81.3%) that did not differ significantly from either stem or leaf petiole/midrib tissue.

Detection of *Rhizoctonia theobromae* is significantly higher in stem tissue compared to leaf midrib, highlighting the stem as the most reliable tissue for real-time PCR-based detection in cassava. Root and leaf tissues do not differ significantly in detection rates.

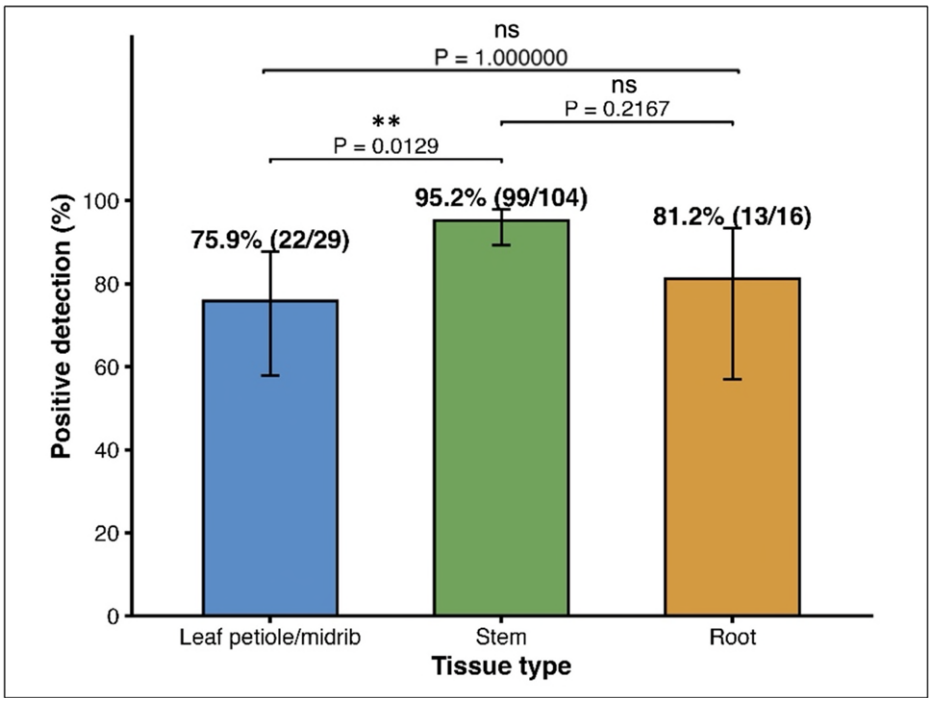


Figure 3. Detection frequencies of *R. theobromae* in leaf petiole/midrib, stem, and root tissues determined using the developed real-time PCR assay. Bars represent the percentage of positive samples for each tissue type. Pairwise comparisons were performed using Fisher's exact test. Asterisks indicate significant differences (**P < 0.01), whereas ns indicates non-significant differences (P > 0.05).

DISCUSSION

This study developed and validated a TaqMan real-time PCR assay targeting a species-specific 28S rDNA region of *R. theobromae*, achieving high sensitivity, and specificity suitable for early detection and diagnosis (Table 1-2; Figure 2). When applied to field-collected cassava, the assay detected the pathogen in leaf, stem, and root tissue of symptomatic plants in high frequencies (81.25-100%), and at low signal intensity, in asymptomatic plants (13.16%) (Table 3). This contributed to the few reported and validated diagnostic tools available for *R. theobromae* associated with CWBD (Oksel & Baysal-Gurel, 2026).

Validation of the real-time PCR assay followed the MIQE 2.0 guidelines (Bustin et al., 2025). Empirical specificity testing was conducted against other fungi co-isolated with *R. theobromae* from CWBD-affected plants such as *Fusarium* sp., *Lasiodiplodia* sp., *Cladosporium* sp., *Alternaria* sp., and unidentified fungal isolates from symptomatic tissue, where there was no observed cross reaction or false result (Table 2). On the other hand, specificity test against other closely-related *Ceratobasidium* species was assessed only *in silico* (Supplementary Table S2) because of the unavailability of culture isolates of *R. theobromae* to support wet-lab cross-reactivity testing. This is because *R. theobromae* is a fastidious, near-obligate hemibiotrophic fungus that is difficult to isolate and maintain in pure culture, as reported in isolation studies cassava and cacao (Gil-Ordóñez et al., 2024; Landicho et al., 2025; McMahon & Purwantara, 2016; Samuels et al., 2012). Other *Ceratobasidium* species are also known to behave not as a pathogen but as an endophyte or beneficial mycorrhizal in some host like orchids (Mosquera-Espinosa et al., 2013) Thus, empirical validation against related *Ceratobasidium* spp. therefore remains an important next step if isolates become available.

The assay's accuracy and sensitivity are comparable to or greater than those of the only currently available CWBD real-time PCR assay, which targets the CaMK/CaMKL locus and was reported to achieve a detection rate of up to 100% and detect as low as 0.003 ng of sample (Silva et al., 2026). Having an independent 28S rDNA-based target is valuable since it gives a second confirmatory marker rather than a variant of the same target locus in the pathogen. This complements established practice in fungal diagnostics, where TaqMan-based real-time PCR assays on conserved ribosomal regions have a corresponding genus- or species-level detection protocol like in other fungal pathogens like as *Colletotrichum* sp. and *Ceratocystis* sp. (Dharmaraj et al., 2022; Phurbu et al., 2026).

In field-level testing, detection rate varied by tissue and growth stage: leaf tissue gave the highest detection rate during the vegetative stage (75.9–95.2%) (Figure 3), whereas stem is the more relevant target at harvest, since it is the material used for propagation and germplasm exchange and its infection status is difficult to assess visually. Despite the high detection rate, testing of planting materials should be done using multiple samples since *R. theobromae* is reported to be tissue localized and maybe unevenly distributed in the host. The RNA *in situ* hybridization (ISH) technology (RNAscope®) study that *R. theobromae* colonizes cassava xylem in a localized, non-uniform distribution, often undetectable in tissue immediately adjacent to clearly infected regions of the same plant (Gil-Ordóñez et al., 2024). This is particularly relevant for asymptomatic samples, where low-intensity signals were observed in some samples, underscoring the need for further evaluation of multi-section sampling for the development of plant material certification protocols. Further testing involving quantification of *R. theobromae* in different plant parts and in stages of the crop is also an important use for this real-time PCR assay in order to further observe colonization pattern of the pathogen. Aside from successful pathogen detection, near-universal amplification of the cassava COX1 internal control indicates it can serve as a co-amplified marker of DNA integrity and a candidate locus for future multiplexing. The negative COX1 result of eight *R. theobromae*-positive leaf samples is maybe due to PCR inhibition or plant DNA degradation, since the midrib/petiole tissues are rich in starch and secondary metabolites and possibly necrotic due to fungal infection (Martin et al., 2025).

The result of this study has two direct implications for phytosanitary measures for this disease. First, given the pathogen's confirmed spread to the Americas, an independent real-time PCR marker strengthens surveillance capacity in newly affected regions (Alves Santos de Oliveira et al., 2025; Oliveira et al., 2026). Second, the assay could support routine screening of asymptomatic planting material source or "mother plant" stock before cuttings are distributed — the typical molecular indexing step prescribed in the FAO/IBPGR guidelines for cassava germplasm movement (Frison & Feliu, 1991). This is because the primary pathway for international spread of *R. theobromae* is through the movement of infected cuttings or plant materials (Pardo et al., 2023). A related and still-open question is whether infested soil acts as an inoculum reservoir, and thus, a sensitive and reliable PCR assay would contributed to testing and screening efforst to understand *R theobromae* epidemiology and spread in the field.

While real-time PCR remains the gold standard for pathogen detection due to its sensitivity, specificity, and accuracy, it is more costly per sample and requires specialized laboratory equipment (Venbrux et al., 2023). However, the real-time PCR assay reported in this study could support the development of field-deployable, point-

of-care diagnostic formats like Loop-mediated Isothermal Amplification (LAMP), Recombinase Polymerase Amplification (RPA), CRISPR/Cas12a fluorescence-based or paper-based lateral flow strip detection systems which are increasingly more useful for field testing and resource-limited laboratories (Anbazhagan et al., 2024; Yang et al., 2025).

CONCLUSION

Real-time PCR assay developed in this study provides a sensitive and specific molecular tool for detection of *R. theobromae* in cassava. The assay consistently detected 10 fg μL^{-1} of purified genomic DNA under the conditions evaluated, showed amplification efficiency close to 100%, and produced no detectable amplification from the non-target fungal isolates tested. Application to field-collected samples demonstrated that *R. theobromae* DNA could be detected in leaf, stem, and root tissues of symptomatic cassava plants, with stem tissues showing the highest overall detection frequency at harvest.

These findings provide a practical basis for improving molecular surveillance of CWBD. In particular, the results suggest that stem tissue is a promising diagnostic sample for symptomatic plants at harvest, whereas leaf petiole/midrib tissue remains a potentially useful and less destructive sample during vegetative growth. Detection in a subset of asymptomatic samples further highlights the potential value of molecular surveillance but requires longitudinal validation before it can be used to predict disease development.

Future studies should focus on validating the assay against a broader collection of *Ceratobasidium* and *Rhizoctonia*-related species, examining longitudinal pathogen detection in asymptomatic plants, determining the spatial localization and viability of the fungus within infected tissues, and investigating the relationship between molecular detection, pathogen abundance, symptom development, and transmission through planting materials. Such studies will be essential for clarifying the role of *R. theobromae* in CWBD and for translating molecular detection into effective disease surveillance and phytosanitary strategies.

SUPPLEMENTARY MATERIALS

Supplementary Table S1. Detection results for *Rhizoctonia theobromae* across sampled cassava plants and tissues**; Supplementary Table S2.** *In silico* evaluation of the *R. theobromae* (syn. *Ceratobasidium theobromae*) TaqMan probe sequence against representative fungal sequences**; Supplementary Table S3**. Sequences and characteristics of primers and probe used in this study**; Supplementary Table S4.** *In-silico* specificity assessment of the *R. theobromae* primer pair using Primer-BLAST**; Supplementary Table S5.** BLASTn analysis of the R. theobromae TaqMan probe sequence**; Supplementary Figure S1.** Representative symptomatic and asymptomatic cassava plants sampled at 6 and 9 months after planting**; Supplementary Figure S2.** Gel electrophoreses image of PCR products obtain from real-time PCR run targeting the R. theobromae 28S rDNA region

AUTHOR CONTRIBUTIONS

Conceptualization, D.M.L., M.S.P. and S.C.; methodology, D.M.L., M.S.P. and S.C.; software, D.M.L.; validation, M.S.P. and S.C.; formal analysis, D.M.L.; investigation, D.M.L., K.R.D.M., J.L.B.V., and M.S.P.; resources, D.M.L. and M.S.P.; data curation, D.M.L., and S.C.; writing—original draft preparation, D.M.L.; writing—review and editing, M.S.P., and S.C.; visualization, D.M.L. and S.C.; supervision, M.S.P. and S.C.; project administration, D.M.L. and S.C.; funding acquisition, D.M.L., M.S.P., and S.C. All authors have read and agreed to the published version of the manuscript.

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