Development of three loop-mediated isothermal amplification (LAMP) assays for the rapid detection of *Calonectria ilicicola*, *Dactylonectria macrodidyma* and the *Dactylonectria* genus in avocado roots

Louisamarie E. Parkinson[1], Duy P. Le[1,2], Elizabeth K. Dann[1]

[1] Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Ecosciences Precinct, 41 Boggo Road, Dutton Park, QLD 4102, Australia

[2] Current address: New South Wales Department of Primary Industries, 21888 Kamilaroi Highway, Narrabri, NSW 2390, Australia

Corresponding author: L. E. Parkinson; E-mail address: l.parkinson@uq.edu.au

Additional Keywords

Black root rot, *Persea americana*, Nectriaceae, nectriaceous fungi, diagnostic

ABSTRACT

Black root rot of avocado is a severe disease of nursery trees and young orchard transplants, causing tree death within a year after planting. In Australia, key pathogens include species complexes *Calonectria ilicicola* and *Dactylonectria macrodidyma*, however several other *Dactylonectria* species also cause the disease. Rapid detection of these pathogens *in planta* is important to faster implement disease management and reduce loss. The purpose of this study was to develop three loop-mediated isothermal amplification (LAMP) diagnostic assays to rapidly identify species within the *C. ilicicola* and *D. macrodidyma* complexes and species in the *Dactylonectria* genus in avocado roots. Primers were designed from β-tubulin sequence data of *C. ilicicola*, and from Histone H3 of *D. macrodidyma* and the *Dactylonectria* genus. The LAMP primers were tested for specificity and sensitivity with 82 fungal isolates, which included the target species complexes, *C. ilicicola* and *D. macrodidyma*; species within the target *Dactylonectria* genus viz. *D. macrodidyma*, *D. anthuriicola*, *D. novozelandica*, *D. pauciseptata* and *D. vitis*; and isolates of non-target species including *Calonectria* sp., *Cylindrocladiella* sp., *Gliocladiopsis forsbergii*, *G. peggii*, *G. whileyi*, *Ilyonectria* sp., *Mariannaea* sp., *Fusarium* sp. and *Phytophthora cinnamomi*. The species-specific LAMP assays were sensitive and specific at DNA concentrations of 1 pg/µl for *C. ilicicola* and 0.01

ng/µl for *D. macrodidyma*, while the *Dactylonectria* genus-wide assay was sensitive to 0.1 ng/µl. Detection of *C. ilicicola* occurred within 10 to 15 min or 15 to 30 min when the template was pure DNA or crude extracts obtained from suspending fungal cultures in sterile water respectively. Detection of *D. macrodidyma* was between 12 to 29 min with pure DNA and 16 to 30 min with crude extracts. *Dactylonectria* spp. were detected within 6 to 25 min with pure DNA and 7 to 23 min with crude extracts. The specificity of the assays was found to be dependent on time and isothermal amplification temperature, with optimal specificity occurring in reactions under 30 minutes and at temperatures of 67°C for *C. ilicicola* and *D. macrodidyma* assays and at 69°C for *Dactylonectria* genus-wide assays. The assays were modified to accommodate a DNA extraction step and use of avocado roots as DNA templates. Detection in avocado roots ranged between 12 to 25 min for *C. ilicicola*, 12 to 26 min for *D. macrodidyma* and 14 to 30 min for species in *Dactylonectria*. The LAMP assays are applicable across multiple agricultural industries as *C. ilicicola*, *D. macrodidyma* and *Dactylonectria* spp. are also important pathogens of various crops and ornamental plants.

INTRODUCTION

Black root rot of avocado caused by soilborne nectriaceous pathogens is an important disease of nursery trees and young orchard transplants, causing tree stunting, wilt, black, rotten and necrotic roots and rapid tree decline and death within a year after planting (Parkinson et al., 2017). In Australia and globally, the important pathogens are *Calonectria ilicicola* (Dann et al., 2012) and *Dactylonectria macrodidyma* (as *Ilyonectria macrodidyma* in Vitale et al., 2012). However more recent studies have demonstrated other species within these genera to cause black root rot, including an undescribed *Calonectria* sp., *D. anthuriicola*, *D. novozelandica* and *D. pauciseptata* (Parkinson et al., 2017). These pathogens are a threat to new plantings and the disease can potentially be undetected in young nursery trees as above ground symptoms of stunting and wilt may not develop until after outplanting (Parkinson et al., 2017). The Australian Avocado Nursery Voluntary Accreditation Scheme (ANVAS) involves regular sampling of nursery material and testing by plant pathologists for a nursery to maintain disease-free accreditation. Standard diagnostic practices include diagnosing disease from symptomatic plant tissue, isolating and culturing the causal agents on selective media and identification of microbial species by microscopy. In instances where a suspected pathogen is difficult to identify with microscopy alone, for example nectriaceous genera which have similar

morphology, molecular techniques have been extremely useful to aid accurate identification. Molecular identification of suspect isolates to species level by isolation, establishing pure cultures, microscopy, DNA extraction, PCR and sequencing, requires time and can take several days to weeks to achieve a diagnosis (Niessen, 2015, Notomi et al., 2015). There is demand for rapid, sensitive and specific molecular methods for early detection of plant diseases (Sankaran et al., 2010) to enable faster implementation of disease management strategies and reduce commercial loss (Fang and Ramasamy, 2015).

Loop-mediated isothermal amplification (LAMP) is a rapid, highly specific and sensitive nucleic acid amplification technique which uses auto-cycling strand displacement DNA synthesis, and produces results from diseased plant tissue in less than 60 minutes (Notomi et al., 2000, Fu et al., 2011). LAMP is catalyzed by the *Bst* DNA polymerase and uses 4 primers which recognize 6 specific sequences on the target DNA (Niessen, 2015). The reaction is initiated by an inner primer, which contains the sense and anti-sense strand sequence of target DNA (Fu et al., 2011), called the F1 and F2 or B1 and B2 sequence (Notomi et al., 2000). These inner primers are called Forward Inner Primer (FIP) and Backward Inner Primer (BIP) (Notomi et al., 2000). A single-stranded synthesized DNA is released by an outer primer (Fu et al., 2011), called the F3 or B3 Primer, and this is used as a template for a second inner and outer primer (Notomi et al., 2000). On the other end of the target sequence, the second inner and outer primers hybridize to the target and produces a stem-loop DNA structure (Fu et al., 2011). In the cycles that follow, one inner primer hybridizes to the loop and initiates displacement DNA synthesis, which produces the original stem-loop structure and a new stem-loop DNA sequence with a stem twice as long (Notomi et al., 2000). The cycling of DNA displacement synthesis results in stem-loop DNA strands with several inverted repeats of the target DNA and loop structures (Notomi et al., 2000). Additional LAMP primers called, Loop Primers (F Loop and B Loop primers), can be added to the reaction to dramatically reduce detection time (Niessen, 2015) and increase the specificity and sensitivity of detection (Nagamine et al., 2002). Loop primers recognize and anneal to target DNA sequences between the F1 and F2 sequence, and the B1 and B2 sequence (Nagamine et al., 2002), providing broader coverage of a length of nucleotide sequences to be detected.

An advantage of LAMP is that the diagnostic is portable and simple to use (Notomi et al., 2015). Since it uses one continuous temperature, the reaction can also be carried out in a water bath or heating block (Notomi et al., 2000) using alternative reaction reagents, enabling cost-

effective options (Fu et al., 2011) and accessibility to developing countries and resource-poor areas (Mori and Notomi, 2009); detection is simplified by visualising salt precipitation or fluorescence in the reaction tube (Tomita et al., 2008). LAMP offers the equivalent or better sensitivity and specificity of PCR (Fukuta et al., 2013, Vincelli and Tisserat, 2008), and the LAMP procedure requires fewer preparation steps (Fu et al., 2011).

LAMP-based diagnostic methods have been developed for various plant pathogens including some nectriaceous fungi such as Boxwood blight fungi, *Calonectria henricotiae* and *C. pseudonaviculata* (Malapi-Wight et al., 2016), and *Fusarium oxysporum* f. sp. *ciceris* of chickpea (Ghosh et al., 2015). So far no LAMP assays exist for detecting *Dactylonectria* spp. LAMP-based assays for detecting *C. ilicicola* and *Dactylonectria* spp. would be extremely useful for the timely identification of causal organisms and disease management for many horticultural, ornamental and field crop industries. For example, *C. ilicicola* also causes crown and root rot of other Lauraceae trees such as bay laurel (Polizzi et al., 2012), collar rot of papaya (Male et al., 2012), leaf spot in ornamental holly (Lechat et al., 2010) and diseases of various field crops including Cylindrocladium black rot of peanut (as *Cylindrocladium parasiticum* in Wright et al., 2010) and crown rot of soybean (Ochi et al., 2011). *D. macrodidyma* causes black foot disease of grapevine (as *Cylindrocarpon macrodidymum* in Halleen et al., 2004), root rot of olive (as *I. macrodidyma* in Úrbez-Torres et al., 2012) and cherimoya (Auger et al., 2015), and is associated with apple seedling replant disease (as *C. macrodidymum* in Tewoldemedhin et al., 2011) and dry root rot of citrus (as *Neonectria macrodidyma* in Adesemoye et al., 2016).

This investigation aimed to develop LAMP assays for the detection of two key nectriaceous pathogens, *C. ilicicola* and *D. macrodidyma*, and a genus-wide assay for detecting *Dactylonectria* spp. in avocado roots. The procedures involved (i) assessing multiple alignments of gene sequence data of a large collection of nectriaceous fungal isolates to identify genes with high nucleotide variation between species, (ii) selecting candidate genes and designing specific LAMP primers from species or genus-unique nucleotide sequences, (iii) demonstrating the specificity, sensitivity and rapid detection of the LAMP assay using DNA extracts, fungal cultures and inoculated plant tissue. The *C. ilicicola* and *D. macrodidyma* isolates used in this study were part of species complexes, each containing potentially new and unresolved species within the phylogenetic clade of the target species (Parkinson, 2017). The LAMP primers were designed to detect all members of the *C. ilicicola* and *D. macrodidyma* species complexes and these were treated as single species in the LAMP assay design.

MATERIALS AND METHODS

Fungal isolates

Two species specific LAMP assays for use with avocado roots were designed for detecting black root rot pathogens, *C. ilicicola* and *D. macrodidyma*, and a genus-wide assay was developed for detecting species in *Dactylonectria*. Eighty-two fungal isolates from the Biosecurity Queensland Plant Pathology Herbarium (BRIP), Department of Agriculture and Fisheries, Brisbane, Queensland, were included this study for demonstrating diagnostic specificity. The isolates tested included a representative number of target species, closely-related species and distantly-related species (Supplementary Table S1).

Designing LAMP primers

Type DNA sequence data of the β-tubulin and histone H3 genes of species in *Calonectria* and *Dactylonectria* were downloaded from Genbank (https://www.ncbi.nlm.nih.gov/). DNA was extracted from BRIP fungal isolates of *Calonectria* and *Dactylonectria* and the β-tubulin and histone H3 gene regions were sequenced following the methods described in Parkinson et al. (2017).

A multiple alignment of the β-tubulin and Histone H3 individual gene regions was performed on each genus in Geneious v 7.1.9 (Biomatters Ltd) (Kearse et al., 2012) using the MAAFT Alignment (Katoh and Standley, 2013) plugin, and included sequence data of fungal species from related genera to identify gene regions unique to the target genus. The β-tubulin gene was selected for designing LAMP primers for *C. ilicicola*, and the histone H3 gene was selected for designing primers for *D. macrodidyma* and *Dactylonectria* spp. as these genes contained high sequence variation between species within the genus to enable target-specific primer annealing. β-tubulin was initially considered for *D. macrodidyma* however there was no sufficient sequence variation to design *D. macrodidyma*-specific LAMP primers.

Unique nucleotide sequences were identified for *C. ilicicola* and *D. macrodidyma* and used as the basis for designing specific LAMP primers and genus-specific *Dactylonectria* primers. For

the *C. ilicicola* and *D. macrodidyma* assays, the outer F3 and B3 LAMP primers were designed from the unique sequences and these were used to generate the Forward Inner Primer (FIP), Backward Inner Primer (BIP), Forward Loop (F Loop) and Backward Loop (B Loop) primers in the LAMP primer designing software, Primer Explorer v 4 (http://primerexplorer.jp) (Table 1). These primer sets were modified manually.

For the genus-wide *Dactylonectria* assay, the consensus sequence of the genus-wide alignment was used as the backbone for designing LAMP primers. Histone H3 sequence data of closely-related genus, *Ilyonectria*, was included to ensure genus-specificity. The F3, B3, FIP, BIP, F Loop and B Loop primers (Table 1) were designed manually from the consensus sequence and the target sequence of each primer was checked in the alignment for specificity to the target genus. Multiple combinations of the LAMP primers were tested in experiments for sensitivity, specificity and optimal isothermal conditions. All LAMP optimization, sensitivity and specificity experiments with pure fungal DNA and crude extracts obtained from suspending fungal cultures in sterile water were conducted once; while LAMP assays with avocado roots were performed twice.

Optimization of LAMP reactions

Experiment 1: Initial sensitivity & specificity testing and optimizing isothermal conditions

Experiment 1 tested the designed primers to confirm primer specificity, to reveal an approximate level of sensitivity for detection and to determine the optimal isothermal temperature at which detection is the fastest. Unless otherwise stated, all isothermal reactions in this study were carried out using OptiGene Isothermal Master Mix and the isothemal block, Genie II supplied by GeneWorks Pty Ltd, Thebarton, South Australia.

The working primer mixture was made to 100 µl containing 1.25 µM F3 primer, 1.25 µM B3 primer, 10 µM FIP primer, 10 µM BIP primer and RNAase free water. The reaction mixture for each test sample contained a total volume of 15 µl, comprized of 2.6 µl RNAase free water, 9 µl OptiGene Isothermal Master Mix, 2.4 µl working primer mixture and 1 µl DNA template of fungal DNA extracts at concentrations ranging from 0.01 pg/µl to 1 ng/µl. The template-free negative control in each LAMP diagnostic test run was RNase-free water.

For the species-specific assays, Genie II was set at 65°C for 60 min, followed by 95 to 80°C annealing and a termination rate of 0.5°C/sec. If sensitivity was demonstrated at a DNA concentration less than 0.01 ng/µl, the experiment was repeated using 63°C and 67°C isothermal amplification temperatures to determine the best isothermal temperature conditions for the LAMP diagnostic. The optimal isothermal temperature was used in subsequent experiments. The *Dactylonectria* genus wide assay used an isothermal amplification temperature of 67°C and the same reaction parameters as the species-specific assays.

In reactions where target species were not detected when DNA concentrations less than 0.01 ng/µl were tested, isothermal temperature comparisons and selection were made in Experiment 2 where loop primers were introduced to the reaction and the optimal LAMP primer combination was determined.

Experiment 2: Testing loop primers for improved detection time, sensitivity and specificity of species-specific LAMP assays

F Loop and B Loop primers were introduced individually or in combination to the LAMP reaction to test for increased reaction speed and improved sensitivity and specificity. The LAMP reagents and sample concentrations were as outlined in Experiment 1, however the working primer mixture now contained 5 µM F Loop primer or 5 µM B Loop primer, or both primers each at 5 µM concentration.

Isothermal amplification for detecting *C. ilicicola* was at 67°C for 60 min, followed by 95 to 80°C annealing and termination at 0.5°C/sec. The test samples for the *D. macrodidyma* diagnostic underwent the same reaction conditions, however the isothermal amplification temperature was set to 65°C. In both diagnostics, the best loop primer combination was selected from comparing the reaction times, sensitivity and specificity, and this primer set was used in further LAMP experiments.

For the *D. macrodidyma* diagnostic, additional experiments which compared isothermal amplification temperatures of 63°C, 65°C and 67°C, were carried out using the selected loop primer set to determine the optimal reaction conditions for this diagnostic (Supplementary Table S3). The selected temperature was used in the following experiments.

In the *Dactylonectria* genus-wide assay, the LAMP reaction parameters were set to 67°C for 45 min followed by 95 to 80°C annealing and termination at 0.5°C/sec (Supplementary Table S3). The LAMP reaction components and master mix were as listed above and underwent further testing for sensitivity, using DNA between 1 ng/µl and 0.1 pg/µl (Supplementary Table S3) following the master mix requirements listed above and isothermal amplification at 67°C for 60 min, then 95 to 80°C annealing and termination at 0.5°C/sec, and then repeated at 69°C for 30 min to test for an improvement in sensitivity.

Experiment 3: Screening DNA from multiple isolates to confirm LAMP specificity

The selected LAMP primer combination and isothermal amplification temperature with DNA templates of a representative number of isolates (target and non-target species) were utilized to thoroughly confirm the specificity of all tests.

For both species-specific diagnostics, the working primer mixture was made to 100 µl containing 1.25 µM F3 primer, 1.25 µM B3 primer, 10 µM FIP primer, 10 µM BIP primer, 5 µM B Loop primer and RNAase free water. The *Dactylonectria* genus-wide assay utilized these reaction components, however 5 µM F Loop primer was also included. Each reaction was prepared as described in Experiment 1, with the fungal DNA template at concentrations of 1 ng/µl or 50 ng/µl and RNase-free water as the template-free negative control (Table 2). For all three assays, the reaction parameters were 67°C isothermal amplification for 30 min, followed by 95 to 80°C annealing and termination at 0.5°C/sec. The *Dactylonectria* genus-wide assay was repeated with the same parameters, and with an additional isothermal amplification temperature of 69°C to test for an improvement in specificity.

LAMP diagnostic detection with fungal cultures and inoculated necrotic avocado tissue

Experiment 4: LAMP diagnostic for detection of target pathogens in fungal cultures

The ability of the diagnostic to detect target DNA in fungal cultures suspended in sterile water was examined. Representative isolates of target and non-target species were grown for 7 to 10 days on half-strength potato dextrose agar amended with 200 ppm streptomycin (sPDA) and incubated at room temperature under black light. Four 0.5 cm^3 plugs of sPDA of each fungal isolate were added to microfuge tubes containing 1 ml sterile distilled water (sd water) and

vigorously shaken to disperse the fungal conidia and mycelia into suspension. One µl of the latter suspension was used as the DNA template in each reaction sample, with one sample containing 1 µl of RNase-free water as the template-free negative control. Some microfuge tubes contained two isolates to represent mixed cultures, with three 0.5 cm^3 plugs of sPDA of each fungal isolate in the tube (Table 3). The LAMP temperature conditions for species-specific diagnostics were set to 67°C isothermal amplification for 30 min, followed by 95 to 80°C annealing and termination at 0.5°C/sec. This was repeated for the *Dactylonectria* genus-wide assay, with an additional isothermal amplification temperature of 69°C.

Experiment 5: Validation of the LAMP diagnostic for detection of target pathogens in inoculated necrotic avocado roots

Experiment 5 tested the LAMP diagnostic for ability to detect the target species directly in avocado root tissue. Twenty-three 6-month-old avocado cv. Reed seedlings were inoculated by amending the potting soil with approximately 250 ml sand:bran:water media mixed with vermiculite (grade 3), prepared as described in Parkinson et al. (2017), and colonized with *C. ilicicola*, *D. macrodidyma*, *D. anthuriicola*, *D. novozelandica*, *D. pauciseptata*, *D. vitis* or a mixture of equal parts (%vol:vol) *C. ilicicola* and one species of the listed *Dactylonectria*. The roots of a healthy, uninoculated 6-month-old cv. Reed seedling were included as a negative control. The plants were maintained in the glasshouse at 22 to 24°C day and 18°C night. At seven weeks post inoculation, the plants were uprooted and assessed for root disease and necrotic or healthy avocado roots were collected for use in the LAMP assay.

The LAMP assays were modified to include a DNA extraction step using the OptiGene Plant DNA Extraction Kit (GeneWorks Pty Ltd, Thebarton, South Australia) for detection with avocado root tissue. For each LAMP assay test sample, necrotic avocado root pieces were sliced into two 1.5 cm sections and added to a 10 ml tube containing a large steel ball bearing from the kit. Each test sample was diluted with three 1.5 cm sections of healthy, uninoculated avocado root pieces. Fungal DNA was extracted following the manufacturer's instructions. One ml Lysis Buffer was added to each tube containing root tissue and the tubes were shaken vigorously by hand for 1 minute to macerate the tissue. Approximately 10 µl of crude DNA extract was transferred by sterile loop into 1 ml dilution buffer and the solution was mixed by inversion. Five µl of diluted crude extract was used as the DNA template in the LAMP reaction.

The LAMP reaction master mix was modified to suit detection with avocado root tissue. The reaction mixture for each test sample contained a total volume of 25 µl, comprized of 15 µl OptiGene Isothermal Master Mix (GeneWorks Pty Ltd, Thebarton, South Australia), 5 µl working primer mixture as listed above and 5 µl DNA template. The isothermal temperature conditions for detecting *C. ilicicola* and *D. macrodidyma* were set to 67°C isothermal amplification for 40 min (Table 4), followed by 95 to 80°C annealing and termination at 0.5°C/sec. This was repeated for the *Dactylonectria* genus-wide assay, with an additional isothermal temperature of 69°C for comparison (Table 4). Each LAMP assay was conducted twice, with freshly sampled roots for each diagnostic run.

RESULTS

Sensitivity and specificity of the LAMP diagnostic, chosen primer sets and optimization of isothermal conditions

Experiment 1: Initial sensitivity & specificity testing and optimizing isothermal conditions

The results of Experiment 1 are listed in Supplementary Table S2. In the initial sensitivity and specificity tests the F3, B3, FIP and BIP primers designed for detecting *C. ilicicola* were sensitive and specific to the target species at DNA concentrations as low as 1 pg/µl. Isothermal amplification temperatures were compared and the optimal temperature was found to be 67°C, enabling detection of 1 ng/µl DNA at 17 min 41 s, with the detection slowing down as the temperature decreased. Isothermal amplification temperature of 67°C was selected for use in all further experiments for detecting *C. ilicicola*. In terms of sensitivity, *C. ilicicola* could be detected at DNA concentrations of 1 pg/µl, however the speed of detection varied and was slower than at higher concentrations; with detection at 24 min 56 s at 65°C and detection at 27 min 24 s at 67°C. In all tests, detection was slowest at isothermal amplification temperature of 63°C.

False positive detection in any sample was determined by an annealing curve not consistent in peak height or position with the curve representing the positive control for detection, an absent annealing curve or an absent annealing and terminating temperature record. Across all assays reliable positive detection occurred under 30 min and any detection after 30 min was considered non-detection.

The F3, B3, FIP and BIP primers designed for detecting *D. macrodidyma* were sensitive and specific to the target species at DNA concentrations of 1 ng/µl but was detected after the 30 min threshold for reliability. The detection of the target species facilitated by these primers was slower and less sensitive than those in the tests with *C. ilicicola* primers. Sensitivity was not demonstrated at DNA concentrations less than 0.1 ng/µl, therefore no further isothermal temperatures were tested for target species, *D. macrodidyma*. Tests with loop primers in Experiment 2 aimed to improve the sensitivity, specificity and detection speed.

Initial specificity testing of F3, B3, FIP and BIP primers for detecting *Dactylonectria* spp. showed detection between 16 min 15 s to 25 min, and non-detection from 30 min. Thirteen out of 14 (~93%) *Dactylonectria* isolates were detected.

Experiment 2: Testing loop primers for improved detection time, sensitivity and specificity

In Experiment 2, combinations of loop primers were tested with the four F3, B3, FIP and BIP primers for an improved speed of detection, sensitivity and specificity for *C. ilicicola*, *D. macrodidyma* or *Dactylonectria* spp. The results of Experiment 2 are listed in Supplementary Table S3.

The B Loop primer in combination with the four F3, B3, FIP and BIP primers performed the best with the highest sensitivity and fastest detection of *C. ilicicola* at low DNA concentrations of 1 pg/µl compared to the other loop primer combinations. The F Loop primer enabled faster detection at DNA concentrations of 1 ng/µl, detecting the DNA 1 min 10s faster than the B Loop primer. However at DNA concentrations less than 0.01 ng/µl, detection with F Loop was slower than the detection with B Loop. Therefore, F Loop and B Loop individually improved detection time and had roughly equal sensitivity, however B Loop was slightly faster at lower DNA concentrations than F Loop. When combined, the F Loop and B Loop primers enabled the fastest detection of DNA in 5 min 51 s to 7 min 5 s at concentrations of 1 to 0.1 ng/µl. However, sensitivity was reduced by using both loop primers as DNA concentrations of 0.01 ng/µl were detected at a similar time to that with B Loop primers alone, and there was no detection at concentrations less than 0.01 ng/µl. Therefore the B Loop primer was selected for use in the LAMP diagnostic for *C. ilicicola* and was tested in subsequent experiments.

The initial tests with the loop primers and four LAMP primers F3, B3, FIP and BIP for the *D. macrodidyma* diagnostic was performed at an isothermal amplification temperature of 65°C, and loop primer combinations were compared to find the optimal primer combination. Including loop primers with the 4 standard LAMP primers improved sensitivity and detection speed, compared to Experiment 1 which had no loop primers. Furthermore, LAMP primers with the B Loop primer, were the most sensitive, detecting target DNA at 0.01 ng/µl within 17 min 7 s. The F Loop primer enabled the fastest detection at 14 min 51 s for concentrations of 1 ng/µl, compared to 16 min 4 s for B Loop and 18 min 2 s for F Loop and B Loop combined. However, despite faster detection, use of the F Loop primer alone resulted in reduced sensitivity, with DNA concentrations detected at concentrations >0.01 ng/µl, compared to successful detection at <0.01 ng/µl with use of the B Loop primer. Use of both loop primers combined resulted in the slowest detection speed and the lowest specificity, with detection occurring between 18 to 19 min and sensitivity to 0.1 ng/µl.

Although sensitivity and detection time was improved with introducing B Loop primers, time appears to be an inhibiting factor in maintaining specificity to the target species. Across all loop primer combinations tested at 65°C, the non-target species, *D. novozelandica*, a close relative of *D. macrodidyma*, was detected within 29 to 55 min, with the F Loop primer detecting non-target species the fastest, and both loop primers combined detecting non-targets the slowest. It was therefore concluded that the success of target-species specificity is dependent on time and the loop primers used. The optimal isothermal amplification time for this diagnostic design was 30 minutes to maintain target species specificity. However the B Loop primer was found to be the most appropriate primer to satisfy the full criteria to a time cut-off for specificity, high sensitivity and adequate detection speed. Therefore the B Loop primer was chosen for subsequent LAMP diagnostic tests.

Further tests in Experiment 2 used the B Loop primer and compared the efficacy of the diagnostic between isothermal amplification temperatures of 63°C, 65°C and 67°C. At DNA concentrations of 1 ng/µl, detection was fastest at 14 min 37 s, with an isothermal temperature of 67°C, and slowed to just over 16 min at 63 and 65°C. Although the greatest sensitivity of detection (at 1 pg/µl DNA) was observed with isothermal temperature of 65°C, the speed of detection was 53 min 20 s, which was beyond the previously determined optimal amplification time of 30 min. At DNA concentrations of 0.01 ng/µl, an isothermal temperature of 67°C

performed detection faster than at 65°C. Therefore, the optimal isothermal temperature of 67°C for *D. macrodidyma* detection was used in all further experiments.

An initial specificity comparison between loop primer combinations was conducted on the *Dactylonectria* genus-wide assay prior to testing the assay for sensitivity. Including any combination of the loop primers in the assay improved the success rate of detection, with 100% of *Dactylonectria* isolates detected within 21 min, compared to 93% in Experiment 1. Including both loop primers resulted in faster detection of targets by approximately 2 minutes on average, compared to using either loop primer alone. All primer combinations resulted in false positive detection of non-target species and negative controls. Specificity was improved by including both loop primers, with detection of non-target *Ilyonectria* sp. (BRIP 53498 a) occurring at 44 min compared to 32 min 15 s and 37 min 45 s with F and B Loop primers, respectively. However since these were detected after 30 min, it was nevertheless considered non-detection rather than true false positive detection.

The full primer set for detecting *Dactylonectria* spp. underwent sensitivity testing at isothermal amplification temperatures of 67°C for 60 minutes and subsequently at 69°C for 30 minutes with representative species from this genus. Two temperatures were tested for improvement in sensitivity. The isothermal amplification time was reduced to 30 min in the second assay as non-specific amplification of template-free samples were found in the first assay at 67°C after 30 min. Detection of target samples after this time was therefore considered a false positive result. The LAMP assay at 67°C for 60 min demonstrated sensitivity averaging 0.1 ng/µl, with *D. macrodidyma* (BRIP 61546a) detection as low as 1 pg/µl. The LAMP assay at 69°C for 30 min demonstrated sensitivity averaging 0.1 ng/µl, although one isolate (*D. anthuriicola* BRIP 60985) could not be detected lower than 1 ng/µl, and another isolate (*D. macrodidyma* BRIP 61546a) was detected at 1 pg/µl but not at 0.01 ng/µl. The LAMP assay at 69°C for 30 min was then tested with non-target *Ilyonectria* isolates in the specificity screening experiment (Experiment 3, Table 2).

LAMP diagnostic for detection of target pathogens in DNA samples, fungal cultures and inoculated necrotic avocado tissue

Experiment 3: Screening DNA samples in isolate collection to confirm LAMP specificity

Experiment 3 confirmed the specificity of the designed LAMP diagnostic design for detecting *C. ilicicola*, *D. macrodidyma* and *Dactylonectria* spp., using 1 ng/µl and 50 ng/µl fungal DNA extracts and fungal mycelia from a representative number of fungal target species, closely-related non-target species and other fungal genera associated with root rot disease.

The *C. ilicicola* LAMP diagnostic design was confirmed to be specific, detecting the target species within 10 to 11 min 30 s at DNA concentrations of 1 ng/µl (Table 2). None of the other isolates in *Calonectria* or the tested genera were detected (Table 2).

The *D. macrodidyma* diagnostic was confirmed to be specific detecting target DNA within 12 min 15 s to 28 min 30 s, (averaging 15 to 16 min), at concentrations of 50 ng/µl. None of the non-target *Dactylonectria* spp. (Table 2) or species in the other genera were detected. Some *D. macrodidyma* isolates were tested twice, at concentrations of 1 ng/µl and 50 ng/µl to observe any differences in detection speed (Table 2). The 50 ng/µl DNA templates were detected considerably faster (by approximately 2 to 9 minutes) than the 1 ng/µl DNA templates.

The *Dactylonectria* genus-wide assay at an isothermal amplification temperature of 67°C was demonstrated to amplify all *Dactylonectria* spp. isolates within 6 to 17 min 30 s (Table 2). However this assay was also found to amplify non-target genera between 9 min 45 s to 25 min 15 s, including one isolate of *C. ilicicola*, *Calonectria* sp., *Gliocladiopsis peggii* and *G. whileyi* and five isolates of *Ilyonectria* sp. (Table 2). A number of false positives was also found in non-target genera detected within approximately 2 min 15 s of the 30 min amplification time (Table 2) and some template-free controls. The alignment of the annealing curve for each sample to the positive control was used to visually assess true positive detection, with false positives denoted by no annealing curve or a short peak compared to the positive control, and amplification of non-target DNA often denoted by annealing curves not aligned to the positive control (with the curve positioned to the left or right of the positive control curve). However considerable care was undertaken when using the curve alignment to decide false or true positives, as large differences in amplification time between *Dactylonectria* species also had an effect on the annealing curve alignment.

The assay at an isothermal amplification temperature of 69°C was demonstrated to have improved specificity, with no detection of isolates in the *Calonectria*, *Cylindrocladiella*, *Gliocladiopsis* or *Mariannaea* genera, or the template-free control (Table 2). Out of the 16

representative isolates of *Ilyonectria* which were selected from 5 different phylogenetic clades within this genus (Parkinson, 2017), only two *Ilyonectria* sp. isolates, BRIP 61090 and BRIP 63711 (BRIP 63711f and BRIP 63711g are duplicate cultures of the same isolate), were detected in this assay (Table 2). These isolates are likely to represent two separate new species, and were recorded only from one sample each in an Australia-wide fungal black root rot disease survey, between 2013 to 2016, of healthy and symptomatic avocado trees, and is thus considered to have a very limited distribution (Parkinson, 2017).

Experiment 4: LAMP diagnostic detection of target pathogens in fungal cultures

Experiment 4 tested the LAMP diagnostic design for detecting *C. ilicicola*, *D. macrodidyma* and *Dactylonectria* spp. using aliquots of sterile distilled water containing fungal mycelia (Table 3).

In the *C. ilicicola* diagnostic (Table 3), all of the target species as pure cultures were detected between 15 to 29 min 15 s, averaging ~16 min for detection. None of the non-target species as pure cultures were detected (Table 3). *C. ilicicola* was detected in 2 out of 3 mixed cultures, with detection occurring between 17 min 49 s and 24 min 5 s (Table 3); the unknown *Calonectria* sp. was not detected.

In the *D. macrodidyma* diagnostic (Table 3), all of the target species as pure cultures were detected within 16 min 30 s to 25 min 15 s. None of the non-target species as pure cultures were detected (Table 3). One hundred percent of mixed cultures containing the target species resulted in positive detection within 16 min 15 s to 29 min 20 s (Table 3). None of the mixed cultures containing only non-target species showed detection (Table 3).

In the *Dactylonectria* genus-wide assay 100% of *Dactylonectria* isolates had positive detection for both isothermal amplification temperatures (Table 3), with detection speeds ranging 6 min 30 s to 25 min 15 s. The detection time varied between the temperatures, suggesting there was no link between detection speed and temperatures from 67 to 69°C. Specificity was demonstrated at 69°C with no detection of *C. ilicicola* at this temperature, compared to non-specific detection at 25 min 15 s at 67°C (Table 3).

Experiment 5: Validation of the LAMP diagnostic detection of target pathogens in inoculated avocado roots

This experiment tested the efficacy of the LAMP diagnostic on target species detection in symptomatic roots of inoculated avocado cv. Reed seedlings. Each LAMP sample tested corresponded to a single plant inoculated with a pathogenic isolate or mixture of isolates (Table 4). Two diagnostic trials were conducted with freshly collected root tissue. Any detection after 30 min was considered to be non-detection and this was confirmed by visual assessment of the annealing curve and temperature record. Any detection from 27 min 30 s was subject to confirmation by visual assessment of annealing curves.

In the *C. ilicicola* assay, across two diagnostic trials, *C. ilicicola* was detected in 100% of plants inoculated with *C. ilicicola* alone, with detection time ranging within 12 min 30 s to 24 min 45 s (Table 4). However *C. ilicicola* in one plant (Plant #23) was not detected in the first trial, but subsequently detected in the second trial. Across two diagnostic trials, eight out of nine plants co-inoculated with *C. ilicicola* had positive detection within 12 min 45 s to 22 min; *C. ilicicola* in Plant #11 was not detected in both trials, and two co-inoculated plants out of nine had positive detection in only one trial (Table 4). Non-detection occurred at 34 min 15 s in one plant (Plant #5) out of 24, and at 36 min 45 s in the *D. macrodidyma* 50 ng/µl DNA sample (Table 4). There was no detection in healthy roots or in template free controls.

In the *D. macrodidyma* assay, across two diagnostic trials, *D. macrodidyma* was detected in 100% of plants inoculated with *D. macrodidyma*, with detection time ranging 12 to 21 min (Table 4). However one plant (Plant #14) was not detected in the first trial, but subsequently detected in the second trial. Across two diagnostic trials, 100% of plants co-inoculated with *D. macrodidyma* were detected within 13 min 30 s to 25 min 30 s. Non-detection of *Dactylonectria* spp. occurred at 39 min 15 s in 3 plants out of 24 (Table 4) across two diagnostic trials. There was no detection in healthy roots or in template free controls.

In the *Dactylonectria* genus-wide assay, across two diagnostic trials for each isothermal amplification temperature tested, *Dactylonectria* was detected in 100% of plants inoculated or co-inoculated with any species of *Dactylonectria* with detection time ranging 13 min 45 s to 28 min 45 s (Table 4) at 67°C; and in 17 out of 19 plants (~89.5%) ranging 9 min 30 s to 29 min 15 s at 69°C. Within this group, 12 out of 19 plants were detected in both of the diagnostic trials at 67°C, and 10 out of 19 plants at 69°C. False negative detection occurred at 69°C in 3 out of 19 plants in Trial 1, and 4 out of 19 plants in Trial 2; at 67°C false negative detection occurred in 4 out of 19 plants in Trial and one in Trial 2. False positive detection of non-target

C. ilicicola occurred in trials of both isothermal temperatures tested, with detection times ranging 17 min 45 s to 28 min in 50 ng/µl DNA samples. In Trial 1 at 67°C 1 out of 4 plants inoculated with *C. ilicicola* alone resulted in false positive detection at 28 min 45 s; in Trial 2 at 67°C 50% of plants inoculated with *C. ilicicola* alone resulted in false positive detection from 27 min 30 s to 27 min 45 s, while the other 50% resulted in non-detection after 30 min. In Trial 1 at 69°C only one plant inoculated with *C. ilicicola* alone resulted in false positive detection at 16 min 45 s. Template free controls and healthy plants resulted in non-detection as indicated by detection after 30 min in all trials at both temperatures, however false positive detection occurred at 27 min 15 s in trial 1 at 67°C.

DISCUSSION

In this study, three LAMP diagnostic assays for detecting *C. ilicicola*, *D. macrodidyma* and species in the *Dactylonectria* genus in avocado roots were developed from β-tubulin (*C. ilicicola*) and Histone H3 (*D. macrodidyma* and *Dactylonectria* spp.) fungal DNA sequence data. Five primers were designed for the species-specific assays, which included one loop primer to increase the specificity, sensitivity and speed of the reaction, allowing reliable detection in avocado roots and fungal DNA of both target species within 30 min. Six primers, including two loop primers, were designed for the *Dactylonectria* genus-wide assay with detection in avocado roots occurring within 30 min. The speed of detection across all assays was fastest and most reliable for DNA extracts, with the detection speed decreasing as fungal cultures and plant tissue were introduced to the diagnostic.

The specificity of the diagnostic for detecting the target species or genus was found to be subject to time and isothermal amplification temperature, with specificity being most reliable in amplifications under 30 minutes and temperatures of 67°C for detecting *C. ilicicola* and *D. macrodidyma* and 69°C for *Dactylonectria* spp. Non-detection was assumed at time points after 30 minutes and any detection after 27 min 30 s was subject to the judgement of the user, in checking for anneal curve consistency with the positive control, in order to rule out false positive or non-detection. Nevertheless non-detection after 30 min were less frequent in the species-specific assays compared to the genus-wide assay. False positives also did not occur under 30 minutes for the species-specific assays, compared to the genus-wide assay, which suggests that the species-specific assays are reliable for *in planta* detection of the target pathogens.

A limitation of the assay is the likelihood of false negatives, with some inoculated root samples in Experiment 5 failing to amplify in one trial, but amplifying in the second trial with a fresh batch of roots (Table 4). Although the majority of inoculated roots resulted in positive detection, the detection in avocado roots was found to be variable and possibly subject to the amount and quality of necrotic root tissue used in each sample. In preliminary work (data not shown) using too much necrotic root tissue (eg. several necrotic roots >1.5 cm) often resulted in false negative detection; however the detection rates improved when fewer roots (up to 2 necrotic roots <1.5 cm) were used and these were also diluted by including a proportion of healthy roots (2 to 4 healthy roots <1.5 cm) in the sample tube for extraction. Perhaps too much necrotic material may have an inhibitory effect on detection and adjusting the concentration of fungal DNA using proportions helped to alleviate these effects. However an accurate concentration of the target fungal DNA in the root tissue could not be determined as the crude extracts contained target fungal DNA, avocado DNA and DNA of soil microbial contaminants. Further testing of the assays in field samples naturally infected with the pathogens should be done to fully validate the diagnostic, as artificial inoculation in the glasshouse may not represent varying pathogen titer in nursery or orchard environments. Nevertheless, the LAMP assays have been used for diagnostic testing of a symptomatic nursery tree submitted through the Australian nursery accreditation scheme and *C. ilicicola* presence was confirmed and supported with identification by fungal isolation. LAMP detection speeds could provide an estimate of the fungal concentration, with faster speeds indicating higher target DNA, however inhibitory compounds in the crude extract may also have an effect on the detection speed or success. Testing multiple samples of a single plant within the assay may also reduce the likelihood of false negative detection in a single diagnostic run.

The genus-wide *Dactylonectria* assay was demonstrated to have efficacy in detecting all *Dactylonectria* species available for testing, with no detection of representative isolates in the *Cylindrocladiella*, *Gliocladiopsis* or *Mariannaea* genera. Two out of the 16 representative isolates of *Ilyonectria* selected within this genus were also detected in this assay (Table 2). *Ilyonectria* is a close relative of *Dactylonectria*, in which the latter genus was separated from the former in a multi-gene phylogeny study (Lombard et al., 2014). In the LAMP primer design phase, *Dactylonectria*, *Ilyonectria* and *Calonectria*, shared multiple conserved regions within each gene examined, rendering it challenging to maintain 100% specificity for all species within *Dactylonectria*, while also excluding all species in the sister genera. A limitation found

in primer design software for designing genus-wide LAMP primers was that the primer combinations produced did not satisfy full exclusion of species in closely-related genera. Manual design of *Dactylonectria* genus-wide primers improved the specificity. However the false positive detection of non-target *C. ilicicola* in under 30 min in the *Dactylonectria* genus-wide assay demonstrated a shortfall in the primer design. For the purpose of the diagnostic assay in detecting multiple species in a known pathogenic genus, the LAMP primers could still be sufficient despite the chance of also detecting non-targets.

Some studies have demonstrated reliable detection without a DNA extraction step, with macerated plant tissue in water used directly as the DNA template (Fukuta et al., 2013). In LAMP studies by Fukuta et al. (2013), tomato roots were cut and placed into 1 ml sd water and the solution was shaken for 1 minute, then 5 µl of the solution was used as the LAMP DNA template for each reaction sample and the LAMP assay was repeated 3 times (Fukuta et al., 2013). Other alternative extraction methods using NaOH and Tris-HCl buffers was outlined by Fukuta et al. (2003) for extraction of DNA from young tomato leaf tissue, which was then used as DNA template in 25 µl LAMP reactions. These methods were tested in preliminary avocado root tissue assays, however there was inconsistent positive detection in inoculated avocado roots (data not shown) and DNA extraction with a commercial kit had improved the efficacy of the assays. DNA extraction from plant material using a Lateral flow device (LFD) (Tomlinson et al., 2010a, Tomlinson et al., 2010b) or with simple buffer solutions (Fukuta et al., 2003) could provide an alternative or potentially reduced-cost DNA extraction prior to the LAMP reaction.

The findings of this study demonstrates the application and adoption of LAMP-based diagnostics in the Australian avocado industry. The simplicity of the testing method enables plant pathology service providers to use the tool as an initial test for confirming presence of important pathogens in nurseries or orchards. Black root rot caused by nectriaceous pathogens poses a significant risk to new plantings in the first year after planting in the field, causing rapid decline and death (Parkinson et al., 2017). Although *C. ilicicola* is infrequently found in Australian avocado nurseries, *Dactylonectria* spp. has a higher prevalence (Parkinson, 2017), and if either are present in the nursery the potential loss in new orchard plantings could be devastating. Use of the LAMP assays for testing nursery trees routinely and prior to dispatching to orchards could potentially prevent industry-wide loss of new plantings in Australia. The LAMP primer sequences are available via publication and the flexibility of the diagnostic

procedure to be used with a range of reagents and equipment enables global accessibility. This diagnostic technology is not limited to testing in avocados as the nectriaceous pathogens of interest are also important pathogens of various crops and ornamental plants, revealing an opportunity for use of this diagnostic tool across multiple agricultural industries around the world.

Acknowledgements

AV14012 was funded by Hort Innovation using the avocado research and development levy and contributions from the Australian Government. Hort Innovation is the grower owned not-for-profit research and development corporation for Australian Horticulture. This project was jointly supported by the Queensland Department of Agriculture and Fisheries (DAF) and the University of Queensland. L. E. Parkinson was a recipient of an Australian Postgraduate Award scholarship. We thank the avocado nursery operators and growers who allowed us to collect samples to build our fungal isolate collection for this study. We also thank Dr Yasushi Ishiguro from the River Basin Research Center, Gifu University, Japan, for their advice on the use of Primer Designer software.

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748

749 **Table 1.** List of primer sequences for each LAMP assay.

Target	**LAMP primer**	**Primer sequence**
C. ilicicola	F3	5'-TGTTGCTGCCCCTGAGCG-3'
	B3	5'GTTACCCTGATCGCGAATGT-3'
	FIP	5'-AGTCAGCAACCTTGTCCTCCGACCGGTTCCGACCGCTTC-3'
	BIP	5'-CTTTCTCAATTCTAGCTCCACCCCAGGGCAGTTTTTGG-3'
	F Loop	5'-TCGTCGAGCTTTGTTGTTGTC-3'
	B Loop	5'-GTCAGTGCGTAAGTGATCATTCC-3'
D. macrodidyma	F3	5'-GTCCACTGGTGGCAAGG-3'
	B3	5'-CACGGAGAGCGACGGTA-3'
	FIP	5'-GTATGGCGATGCATTTTTGATCTTCCAAGGCTGGTGAGT-3'
	BIP	5'-ACCTTAACCATCAACAGCCCGCGTAGCGGTGAGGCTTCTTG-3'
	F Loop	5'-CGCGACGTGTCAAGTAAATGG-3'
	B Loop	5'-GCCCCCTCTACCGGTGGTGT-3'
Dactylonectria spp.	F3	5'-TCCAAGGCTGGTGAGTCTCG-3'
	B3	5'-ACTCACGAGACGCTGGAA-3'
	FIP	5'-GCTCTTGCGGGCTGTTGATATTTACTTGACGCGTCGC-3'
	BIP	5'-TCAAGAAGCCTCACCGCTACATGAGGAGCTCGGTCGACT-3'
	F Loop	5'-GGTTGAGGTTAGTATGGCGATG-3'
	B Loop	5'-TACCGTCGCTCTCCGTGA-3'

750

751 **Table 2.** Experiment 3 – Screening DNA from multiple isolates to confirm LAMP specificity of optimized primer
752 sets and isothermal conditions for detection of target species, *C. ilicicola*, *D. macrodidyma* and *Dactylonectria*
753 spp. using pure fungal DNA extracts as templates.

LAMP assay	Isolate (BRIP) ID	Species	DNA concn (ng/µl)	Detection time at 67°C (min:s)	Detection time at 69°C (min:s)
C. ilicicola	54018 a	*C. ilicicola*	1	10:22	
	60982	*C. ilicicola*	1	10:05	
	60992	*C. ilicicola*	1	11:12	
	61291	*C. ilicicola*	1	10:34	
	60388	*C. ilicicola*	1	10:51	
	60389	*C. ilicicola*	1	11:15	
	60397	*C. ilicicola*	1	10:45	
	53933 a	*C. ilicicola*	1	11:35	
	55531 a	*C. ilicicola*	1	10:21	
	53653 a	*C. ilicicola*	1	10:53	
	61448	*Calonectria* sp.	1	ND	
	60981	*Calonectria* sp.	1	ND	
	63712	*Calonectria* sp.	1	ND	
	15920 a	*Calonectria* sp.	1	ND	
	16747 a	*Calonectria* sp.	1	ND	
	60986	*Cylindrocladiella* sp.	1	ND	
	61292	*Cylindrocladiella* sp.	1	ND	
	62001 a	*D. macrodidyma*	1	ND	
	62000 a	*D. novozelandica*	1	ND	
	61428 b	*D. pauciseptata*	1	ND	
	61429 b	*D. anthuriicola*	1	ND	
	n/a	*Fusarium* sp.	1	ND	
	60983	*G. peggii*	1	ND	
	61430	*G. whileyi*	1	ND	
	61349 d	*Ilyonectria* sp.	1	ND	
	53498 a	*Ilyonectria* sp.	1	ND	
	63711 e	*Mariannaea* sp.	1	ND	
	61192 c	*Mariannaea* sp.	1	ND	
	n/a	RNase-free water	0	ND	

D. macrodidyma	60979	*D. macrodidyma*	50	15:22
	61294 a	*D. macrodidyma*	50	14:17
	61431 c	*D. macrodidyma*	50	13:44
	61434 a	*D. macrodidyma*	50	18:54
	61436 a	*D. macrodidyma*	50	14:05
	60907 b	*D. macrodidyma*	50	14:31
	61090 c	*D. macrodidyma*	50	12:16
	61354 c	*D. macrodidyma*	50	14:25
	61195 d	*D. macrodidyma*	50	13:18
	61306 a	*D. macrodidyma*	50	13:48
	61442	*D. macrodidyma*	50	28:30
	61444 a	*D. macrodidyma*	50	13:31
	62000 b	*D. macrodidyma*	50	26:05
	62000 g	*D. macrodidyma*	50	25:02
	62001 b	*D. macrodidyma*	50	15:48
	62001 a	*D. macrodidyma*	1	15:29
	62001 a	*D. macrodidyma*	50	13:18
	62002 a	*D. macrodidyma*	1	15:34
	62002 a	*D. macrodidyma*	50	12:40
	61546 a	*D. macrodidyma*	1	17:15
	61546 a	*D. macrodidyma*	50	12:32
	62005 a	*D. macrodidyma*	1	26:40
	62005 c	*D. macrodidyma*	50	16:51
	61428 b	*D. pauciseptata*	1	ND
	61428 c	*D. pauciseptata*	1	ND
	62000 c	*D. novozelandica*	1	ND
	61433 a	*D. pauciseptata*	50	ND
	52550 a	*D. pauciseptata*	50	ND
	60991 a	*D. pauciseptata*	50	ND
	63707 a	*D. pauciseptata*	50	ND
	63713	*D. pauciseptata*	50	ND
	62000 d	*D. novozelandica*	1	ND
	61429 b	*D. anthuriicola*	50	ND
	61195 b	*D. vitis*	50	ND
	63708 b	*D. vitis*	50	ND
	61263 g	*D. vitis*	50	ND
	54018 a	*C. ilicicola*	1	ND
	60986	*Cylindrocladiella* sp.	1	ND
	n/a	*Fusarium* sp.	1	ND

	60983	*G. peggii*	1	ND	
	61349 a	*G. forsbergii*	50	ND	
	53498 a	*Ilyonectria* sp.	1	ND	
	61192 c	*Mariannaea* sp.	1	ND	
	n/a	*Phytophthora cinnamomi*	>50	ND	
	n/a	RNase-free water	0	ND	
Dactylonectria spp.	60979	*D. macrodidyma*	50	10:15	9:00
	61349 e	*D. macrodidyma*	50	7:30	7:45
	61431 c	*D. macrodidyma*	50	7:30	7:15
	61434 a	*D. macrodidyma*	50	11:45	11:30
	61436 a	*D. macrodidyma*	50	7:30	7:15
	60907 b	*D. macrodidyma*	50	8:45	8:15
	61090 c	*D. macrodidyma*	50	7:00	7:00
	61354 c	*D. macrodidyma*	50	8:00	8:00
	61195 d	*D. macrodidyma*	50	7:30	7:15
	61306 a	*D. macrodidyma*	50	8:00	8:00
	61442	*D. macrodidyma*	50	9:45	9:00
	61444 a	*D. macrodidyma*	50	8:00	7:45
	62000 b	*D. macrodidyma*	50	12:45	11:30
	62000 g	*D. macrodidyma*	50	13:15	12:30
	n/a	RNase-free water	0	ND	ND
	62001 b	*D. macrodidyma*	50	11:00	10:30
	62001 a	*D. macrodidyma*	50	6:15	7:00
	62002 a	*D. macrodidyma*	50	6:00	6:45
	61546 a	*D. macrodidyma*	50	6:00	7:00
	62005 c	*D. macrodidyma*	50	7:15	8:45
	61295 d	*D. pauciseptata*	50	12:15	12:00
	61428 b	*D. pauciseptata*	50	12:30	13:15
	61428 c	*D. pauciseptata*	50	13:30	14:00
	61428 d	*D. pauciseptata*	50	12:15	12:30
	61433 a	*D. pauciseptata*	50	13:30	13:45
	52550 a	*D. pauciseptata*	50	17:30	16:30
	60991 a	*D. pauciseptata*	50	11:30	11:30
	63707 a	*D. pauciseptata*	50	13:30	13:15
	63713	*D. pauciseptata*	50	13:15	13:15
	62000 a	*D. novozelandica*	50	11:00	12:30
	n/a	RNase-free water	0	26:30 FP	ND
	62000 c	*D. novozelandica*	50	12:00	13:00
	62000 d	*D. novozelandica*	50	11:30	16:00

60985	*D. anthuriicola*	50	11:15	15:15
61429 b	*D. anthuriicola*	50	11:00	15:00
61306 b	*D. anthuriicola*	50	11:45	15:15
61437 b	*D. anthuriicola*	50	9:15	15:30
61195 b	*D. vitis*	50	10:00	12:00
63708 b	*D. vitis*	50	9:00	12:15
61263 f	*D. vitis*	50	10:30	11:45
61263 g	*D. vitis*	50	6:15	12:45
61352 c	*D. macrodidyma*	50	9:15	8:00
61354 c	*D. macrodidyma*	50	8:00	10:30
53498 a	*Ilyonectria* sp.	50	29:15 FP	ND
60980	*Ilyonectria* sp.	50	28:30 FP	ND
61349 d	*Ilyonectria* sp.	50	ND	ND
n/a	RNase-free water	0	ND	ND
61546 a	*D. macrodidyma*	50	6:15	7:15
61263 f	*D. vitis*	50	9:15	11:00
61294 a	*D. macrodidyma*	50	7:30	9:00
54018 a	*C. ilicicola*	50	21:00 FP	ND
60982	*C. ilicicola*	50	29:15 FP	ND
61448	*Calonectria* sp.	50	24:00 FP	ND
60981	*Calonectria* sp.	50	29:15 FP	ND
60986	*Cylindrocladiella* sp.	50	ND	ND
61292	*Cylindrocladiella* sp.	50	ND	ND
63711 e	*Mariannaea* sp.	50	29:15 FP	ND
61192 c	*Mariannaea* sp.	50	ND	ND
60983	*G. peggii*	50	21:30 FP	ND
61430	*G. whileyi*	50	21:45 FP	ND
63711 f	*Ilyonectria* sp.	50	20:30 FP	17:00 FP
61090 a	*Ilyonectria* sp.	50	25:15 FP	ND
n/a	RNase-free water	0	29:15 FP	ND
61546 a	*D. macrodidyma*	50	9:15	7:30
60991 a	*D. pauciseptata*	50	12:00	12:30
61432 b	*Ilyonectria* sp.	50	27:45 FP	ND
61090 a	*Ilyonectria* sp.	50	25:15 FP	28:30 FP
63711 f	*Ilyonectria* sp.	50	9:45 FP	15:30 FP
63711 g	*Ilyonectria* sp.	50	18:00 FP	17:30 FP
61546 i	*Ilyonectria* sp.	50	ND	ND
61194 a	*Ilyonectria* sp.	50	25:30 FP	ND
61435 c	*Ilyonectria* sp.	50	29:00 FP	ND

62004 b	*Ilyonectria* sp.	50	ND	ND
60989	*Ilyonectria* sp.	50	ND	ND
61443	*Ilyonectria* sp.	50	29:15 FP	ND
61293	*Ilyonectria* sp.	50	ND	ND
53652 a	*Ilyonectria* sp.	50	29:15 FP	ND
61303 d	*Ilyonectria* sp.	50	29:15 FP	ND
n/a	RNase-free water	0	ND	ND

754 ND not detected

755 FP false positive as indicated by detection of a non-target species in under 30 minutes or an inconsistent or
756 absent annealing curve.

757

758

759 **Table 3.** Experiment 4 – Testing optimized primer sets and isothermal conditions for specificity in detection of
760 target species, *C. ilicicola*, *D. macrodidyma* and *Dactylonectria* spp. using crude extracts from a suspension of
761 fungal cultures in sterile distilled water.

LAMP assay	Isolate (BRIP) ID	Species	DNA concn (ng/µl)	Detection time at 67°C (min:s)	Detection time at 69°C (min:s)
C. ilicicola	54018 a	*C. ilicicola*	Undetermined	15:15	
	60982	*C. ilicicola*	Undetermined	15:00	
	60992	*C. ilicicola*	Undetermined	15:48	
	61291	*C. ilicicola*	Undetermined	16:10	
	61291	*C. ilicicola*	Undetermined	17:28	
	60389	*C. ilicicola*	Undetermined	29:15	
	53933 a	*C. ilicicola*	Undetermined	23:16	
	60981	*Calonectria* sp.	Undetermined	ND	
	54018 a + 60981	Mixed culture: *C. ilicicola* + *Calonectria* sp.	Undetermined	ND	
	60982 + 60981	Mixed culture: *C. ilicicola* + *Calonectria* sp.	Undetermined	24:05	
	61291 + 60981	Mixed culture: *C. ilicicola* + *Calonectria* sp.	Undetermined	17:49	
	n/a	*Ilyonectria* sp. fungal culture	Undetermined	ND	
	n/a	RNase-free water	0	ND	
D. macrodidyma	61294 a	*D. macrodidyma*	Undetermined	18:16	
	61294 a	*D. macrodidyma*	Undetermined	17:27	
	61349 e	*D. macrodidyma*	Undetermined	16:35	
	61349 e	*D. macrodidyma*	Undetermined	22:38	
	62001 b	*D. macrodidyma*	Undetermined	16:28	
	62001 b	*D. macrodidyma*	Undetermined	25:14	
	61428 d	*D. pauciseptata*	Undetermined	ND	
	62000 d	*D. novozelandica*	Undetermined	ND	
	60985	*D. anthuriicola*	Undetermined	ND	
	61294 a + 62000 d	*D. macrodidyma* + *D. novozelandica*	Undetermined	16:13	
	61349 e + 62000 d	*D. macrodidyma* + *D. novozelandica*	Undetermined	29:21	
	62001 b + 62000 d	*D. macrodidyma* + *D. novozelandica*	Undetermined	20:41	

	61428 d + 62000 d	*D. pauciseptata* + *D. novozelandica*	Undetermined	ND	
	n/a	*Ilyonectria* sp. fungal culture	Undetermined	ND	
	n/a	RNase-free water	0	ND	
Dactylonectria spp.	61546 a	*D. macrodidyma*	Undetermined	6:30	7:15
	61349 e	*D. macrodidyma*	Undetermined	17:00	12:15
	61429 b	*D. macrodidyma*	Undetermined	20:15	19:00
	60985	*D. anthuriicola*	Undetermined	17:00	16:00
	62000 d	*D. novozelandica*	Undetermined	13:15	14:15
	61428 d	*D. pauciseptata*	Undetermined	20:30	17:15
	63708 b	*D. vitis*	Undetermined	13:15	14:45
	61349 e + 54018 a	*D. macrodidyma* + *C. ilicicola*	Undetermined	14:15	10:30
	61429 b + 54018 a	*D. macrodidyma* + *C. ilicicola*	Undetermined	19:45	23:00
	60985 + 54018 a	*D. anthuriicola* + *C. ilicicola*	Undetermined	16:45	18:15
	62000 d + 54018 a	*D. novozelandica* + *C. ilicicola*	Undetermined	25:15	18:30
	61428 d + 54018 a	*D. pauciseptata* + *C. ilicicola*	Undetermined	16:30	21:45
	63708 b + 54018 a	*D. vitis* + *C. ilicicola*	Undetermined	20:15	20:00
	54018 a	*C. ilicicola*	Undetermined	25:15 FP	ND
	n/a	RNase-free water	0	ND	ND

762 ND not detected.

763 FP false positive as indicated by detection of a non-target species in under 30 min or an inconsistent or absent
764 annealing curve.

765 **Table 4.** Experiment 5 – Validation of the LAMP diagnostic for detection of target pathogens, *C. ilicicola*, *D. macrodidyma* and *Dactylonectria* spp. in inoculated necrotic
766 avocado roots.

Plant number	**Isolate (BRIP) ID**	**Inoculum**	**Detection of *C. ilicicola***		**Detection of *D. macrodidyma***		**Detection of *Dactylonectria* spp.**			
			Trial 1 at 67°C (min:s)	**Trial 2 at 67°C (min:s)**	**Trial 1 at 67°C (min:s)**	**Trial 2 at 67°C (min:s)**	**Trial 1 at 67°C (min:s)**	**Trial 2 at 67°C (min:s)**	**Trial 1 at 69°C (min:s)**	**Trial 2 at 69°C (min:s)**
n/a	62001 a	*D. macrodidyma* (50 ng/µl DNA)	ND	ND	7:45	8:00	5:30	5:15	5:45	7:30
n/a	54018 a	*C. ilicicola* (50 ng/µl DNA)	7:45	7:45	ND	ND	ND	28:00 FP	36:15 ND	34:45 ND
1	60985	*D. anthuriicola*	ND	ND	ND	ND	16:00	15:30	18:15	18:45
2	60985	*D. anthuriicola*	ND	ND	ND	ND	15:00	13:45	22:45	29:45
3	62000 d	*D. novozelandica*	ND	ND	39:15 ND	ND	15:30	39:15 ND	ND	22:30
4	62000 d	*D. novozelandica*	ND	ND	ND	ND	17:30	19:45	39:15 ND	28:45
5	61428 d	*D. pauciseptata*	34:15 ND	ND	ND	39:15 ND	20:00	28:45	ND	29:15
6	61428 d	*D. pauciseptata*	ND	ND	ND	ND	18:15	20:00	18:45	39:15 ND
7	63708 b	*D. vitis*	ND	ND	ND	ND	20:00	15:00	20:45	16:30
8	63708 b	*D. vitis*	ND	ND	ND	ND	16:00	15:15	17:30	17:30
9	60985 + 54018 a	*D. anthuriicola* + *C. ilicicola*	13:00	15:45	ND	ND	17:15	15:15	18:45	30:45 ND
10	62000 d + 54018 a	*D. novozelandica* + *C. ilicicola*	14:45	15:15	ND	ND	ND	20:30	ND	ND
11	61428 d + 54018 a	*D. pauciseptata* + *C. ilicicola*	35:15 ND	36:00 ND	ND	ND	20:15	16:15	27:00 FP	ND

12	63708 b + 54018 a	*D. vitis* + *C. ilicicola*	13:45	ND	ND	39:15 ND	23:30	20:15	31:45 ND	ND
13	n/a	Uninoculated	ND	ND	ND	ND	30:30 ND	36:30 ND	33:15 ND	39:15 ND
n/a	n/a	Template free	ND	ND	ND	ND	30:30 ND	32:45 ND	39:15 ND	39:15 ND
n/a	62001 a	*D. macrodidyma* (50 ng/µl DNA)	ND	36:45 ND	8:00	8:00	15:15	5:45	7:45	8:00
n/a	54018 a	*C. ilicicola* (50 ng/µl DNA)	6:15	6:30	ND	ND	20:30 FP	24:00 FP	17:45 FP	39:15 ND
14	61349 e	*D. macrodidyma*	ND	ND	ND	21:00	ND	13:45	22:00	ND
15	61349 e	*D. macrodidyma*	ND	ND	12:00	13:45	10:45	29:00 FP	14:00	9:30
16	61349 e + 54018 a	*D. macrodidyma* + *C. ilicicola*	22:00	12:30	24:45	23:00	29:15 FP	20:00	15:15	28:00
17	61349 e + 54018 a	*D. macrodidyma* + *C. ilicicola*	13:15	11:45	13:30	18:45	15:15	16:45	16:30	18:00
18	61349 e + 54018 a	*D. macrodidyma* + *C. ilicicola*	17:30	15:15	15:15	19:00	ND	15:30	18:45	21:15
19	61349 e + 54018 a	*D. macrodidyma* + *C. ilicicola*	16:15	12:45	20:45	16:15	14:15	13:45	18:00	14:00
20	61349 e + 54018 a	*D. macrodidyma* + *C. ilicicola*	27:15 FP	15:00	25:30	18:15	ND	14:15	14:00	18:00
21	54018 a	*C. ilicicola*	17:00	14:30	ND	ND	28:45 FP	27:45 FP	ND	ND
22	54018 a	*C. ilicicola*	21:00	24:45	ND	ND	ND	27:30 FP	39:15 ND	ND
23	54018 a	*C. ilicicola*	ND	16:15	ND	ND	ND	37:00 ND	16:45 FP	39:00 ND
24	54018 a	*C. ilicicola*	12:30	13:45	ND	ND	ND	35:45 ND	ND	ND
n/a	n/a	Template free	ND	ND	ND	ND	27:15 FP	38:15 ND	39:15 ND	39:15 ND

767 ND not detected or non-detection as indicated by detection after 30 minutes.

768 FP false positive as indicated by detection of a non-target species in under 30 min or an inconsistent or absent annealing curve.

Supplementary Table S1. List of fungal isolates used as DNA templates in each experiment of this study.

BRIP ID	Species name	Experiment number				
54018 a	*Calonectria ilicicola*	1	2	3	4	5
60982	*C. ilicicola*			3	4	
61291	*C. ilicicola*			3	4	
55531 a	*C. ilicicola*			3		
53653 a	*C. ilicicola*			3		
60992	*C. ilicicola*			3	4	
60388	*C. ilicicola*			3		
60389	*C. ilicicola*			3	4	
60397	*C. ilicicola*			3		
53933 a	*C. ilicicola*			3	4	
61448	*Calonectria* sp.			3		
60981	*Calonectria* sp.	1	2	3	4	
63712	*Calonectria* sp.			3		
16747 a	*Calonectria* sp.			3		
15920 a	*Calonectria* sp.			3		
62001 a	*Dactylonectria macrodidyma*	1	2	3		5
62002 a	*D. macrodidyma*			3		
61546 a	*D. macrodidyma*	1	2	3	4	
62000b	*D. macrodidyma*			3		
62000 g	*D. macrodidyma*			3		
62005 a	*D. macrodidyma*			3		
60979	*D. macrodidyma*			3		
61431 c	*D. macrodidyma*			3		
61434 a	*D. macrodidyma*			3		
61436 a	*D. macrodidyma*			3		
60907 b	*D. macrodidyma*			3		
61090 c	*D. macrodidyma*			3		
61195 d	*D. macrodidyma*			3		
61306 a	*D. macrodidyma*			3		
61442	*D. macrodidyma*			3		
61444 a	*D. macrodidyma*			3		
62005 c	*D. macrodidyma*			3		
61294 a	*D. macrodidyma*			3	4	
62001 b	*D. macrodidyma*			3	4	
61352 c	*D. macrodidyma*	1	2	3		
61354 c	*D. macrodidyma*			3		
61349 e	*D. macrodidyma*	1	2	3	4	5
61295 d	*D. pauciseptata*	1	2	3		

61428 b	*D. pauciseptata*			3		
61428 c	*D. pauciseptata*			3		
61428 d	*D. pauciseptata*	1	2	3	4	5
61433 a	*D. pauciseptata*	1	2	3		
60991 a	*D. pauciseptata*	1	2	3		
52550 a	*D. pauciseptata*			3		
63707 a	*D. pauciseptata*			3		
63713	*D. pauciseptata*	1	2	3		
62000 a	*D. novozelandica*	1	2	3		
62000 c	*D. novozelandica*	1	2	3		
62000 d	*D. novozelandica*	1	2	3	4	5
61429 b	*D. anthuriicola*	1	2	3	4	
60985	*D. anthuriicola*	1	2	3	4	5
61306 b	*D. anthuriicola*			3		
61437 b	*D. anthuriicola*			3		
63708 b	*D. vitis*			3	4	5
61195 b	*D. vitis*			3		
61263 f	*D. vitis*	1	2	3		
61263 g	*D. vitis*			3		
60986	*Cylindrocladiella* sp.			3		
61292	*Cylindrocladiella* sp.			3		
62004 a	*Fusarium* sp.			3		
60983	*Gliocladiopsis peggii*			3		
61430	*G. whileyi*			3		
61349 a	*G. forsbergii*			3		
61349 d	*Ilyonectria* sp.			3		
53498 a	*Ilyonectria* sp.	1	2	3		
60980	*Ilyonectria* sp.			3		
61090 a	*Ilyonectria* sp.			3		
63711 f	*Ilyonectria* sp.			3		
63711 g	*Ilyonectria* sp.			3		
61546 i	*Ilyonectria* sp.			3		
61194 a	*Ilyonectria* sp.			3		
61435 c	*Ilyonectria* sp.			3		
62004 b	*Ilyonectria* sp.			3		
60989	*Ilyonectria* sp.			3		
61443	*Ilyonectria* sp.			3		
61293	*Ilyonectria* sp.			3		
53652 a	*Ilyonectria* sp.			3		
61303 d	*Ilyonectria* sp.			3		

n/a	*Ilyonectria* sp. (fungal culture only)	4
63711 e	*Mariannaea* sp.	3
61192 c	*Mariannaea* sp.	3
n/a	*Phytophthora cinnamomi* (fungal culture only)	3

Where experiment number refers to:

1. Experiment 1: Initial sensitivity & specificity testing and optimising isothermal conditions
2. Experiment 2: Testing loop primers for improved detection time, sensitivity and specificity
3. Experiment 3: Screening DNA samples in isolate collection to confirm LAMP specificity
4. Experiment 4: LAMP diagnostic detection in fungal cultures
5. Experiment 5: LAMP diagnostic detection in necrotic inoculated avocado root tissue

Supplementary Table S2. Experiment 1 – Initial sensitivity and specificity testing and optimizing isothermal conditions of the LAMP assay design for detecting *C. ilicicola, D. macrodidyma* and *Dactylonectria* spp. with F3, B3, FIP and BIP primers.

LAMP assay	Isolate (BRIP) ID	Species	DNA concn	Detection time at isothermal reaction temperature (min:s)		
				63°C	65°C	67°C
C. ilicicola (sensitivity testing)	54018 a	*C. ilicicola*	1 ng/µl	20:22	19:50	17:41
			0.1 ng/µl	21:29	18:24	19:34
			0.01 ng/µl	29:17	20:57	26:45
			1 pg/µl	47:47	24:56	27:24
				ND		
			0.1 pg/µl	ND	ND	ND
			0.01 pg/µl	ND	ND	ND
	60981	*Calonectria* sp.	1 ng/µl	ND	ND	ND
	n/a	RNase-free water	0 ng/µl	ND	ND	ND
D. macrodidyma (sensitivity testing)	62001 a	*D. macrodidyma*	1 ng/µl		32:00 ND	
			0.1 ng/µl		43:00 ND	
			0.01 ng/µl		ND	
			1 pg/µl		ND	
			0.1 pg/µl		ND	
			0.01 pg/µl		ND	
	62000 a	*D. novozelandica*	1 ng/µl		ND	
	n/a	RNase-free water	0 ng/µl		ND	
Dactylonectria spp. (specificity testing)	61546 a	*D. macrodidyma*	50 ng/µl			16:15
	63713	*D. pauciseptata*	50 ng/µl			25:00
	60991 a	*D. pauciseptata*	50 ng/µl			19:30
	61433 a	*D. pauciseptata*	50 ng/µl			23:15
	62001 a	*D. macrodidyma*	50 ng/µl			18:00
	61428 d	*D. pauciseptata*	50 ng/µl			21:45
	62000 d	*D. novozelandica*	50 ng/µl			39:15 ND
	60985	*D. anthuriicola*	50 ng/µl			23:15
	61295 d	*D. pauciseptata*	50 ng/µl			20:30
	61263 f	*D. vitis*	50 ng/µl			19:30
	61429 b	*D. anthuriicola*	50 ng/µl			22:45
	61349 e	*D. macrodidyma*	50 ng/µl			18:45
	61352 c	*D. macrodidyma*	50 ng/µl			17:30
	62000 c	*D. novozelandica*	50 ng/µl			30:00
	53498 a	*Ilyonectria* sp.	50 ng/µl			39:45 ND
	n/a	RNase-free water	0 ng/µl			38:30 ND

4 ND not detected or non-detection as indicated by detection after 30 minutes.

Supplementary Table S3. Experiment 2 – Sensitivity and specificity testing of F3, B3, FIP, BIP, F Loop and B Loop primers and isothermal reaction temperatures for improved time, sensitivity and specificity for detecting *C. ilicicola, D. macrodidyma* and *Dactylonectria* spp.

LAMP assay	Isolate (BRIP) ID	Species	DNA concn	Detection time at isothermal amplification temperature (min:s)							
				F Loop primer		B Loop primer			F & B Loop primer		
				65°C	67°C	63°C	65°C	67°C	65°C	67°C	69°C
C. ilicicola (sensitivity testing)	54018 a	*C. ilicicola*	1 ng/µl		9:09			10:31		5:51	
			0.1 ng/µl		11:14			12:24		7:05	
			0.01 ng/µl		16:07			14:44		14:13	
			1 pg/µl		26:58			14:54		ND	
			0.1 pg/µl		ND			ND		ND	
	60981	*Calonectria* sp.	1 ng/µl		ND			ND		ND	
	n/a	RNase-free water	0 ng/µl		ND			ND		ND	
D. macrodidyma (sensitivity testing)	62001 a	*D. macrodidyma*	1 ng/µl	14:51		16:06	16:04	14:37	18:02		
			0.1 ng/µl	15:59		18:36	19:43	22:32	19:47		
			0.01 ng/µl	26:09		17:07	36:06 ND	18:48	ND		
			1 pg/µl	ND		ND	53:20 ND	ND	ND		
			0.1 pg/µl	ND		ND	ND	ND	ND		
			0.01 pg/µl	ND		ND	ND	ND	ND		
	62000 a	*D. novozelandica*	1 ng/µl	28:57		33:54 ND	34:43 ND	30:26 ND	54:28 ND		
	n/a	RNase-free water	0 ng/µl	ND		ND	ND	ND	ND		
Dactylonectria spp. (specificity testing)	61546 a	*D. macrodidyma*	50 ng/µl		9:00			8:45		6:45	
	63713	*D. pauciseptata*	50 ng/µl		20:30			13:00		13:30	
	60991 a	*D. pauciseptata*	50 ng/µl		18:15			11:45		12:00	
	61433 a	*D. pauciseptata*	50 ng/µl		20:30			13:45		13:45	
	62001 a	*D. macrodidyma*	50 ng/µl		8:45			8:45		6:30	
	61428 d	*D. pauciseptata*	50 ng/µl		18:45			12:45		13:15	

	62000 d	*D. novozelandica*	50 ng/µl	17:00	13:15	14:30	
	60985	*D. anthuriicola*	50 ng/µl	21:00	12:30	13:00	
	61295 d	*D. pauciseptata*	50 ng/µl	18:00	12:15	12:30	
	61263 f	*D. vitis*	50 ng/µl	17:45	9:15	10:15	
	61429 b	*D. anthuriicola*	50 ng/µl	20:30	12:45	13:00	
	61349 e	*D. macrodidyma*	50 ng/µl	9:30	9:45	7:15	
	61352 c	*D. macrodidyma*	50 ng/µl	8:45	9:00	6:45	
	62000 c	*D. novozelandica*	50 ng/µl	14:00	11:00	11:15	
	53498 a	*Ilyonectria* sp.	50 ng/µl	32:15	37:45	44:00	
				ND	ND	ND	
	n/a	RNase-free water	0 ng/µl	34:15	32:30	39:45	
				ND	ND	ND	
Dactylonectria spp. (sensitivity testing)	61546 a	*D. macrodidyma*	1 ng/µl			9:00	10:30
			0.1 ng/µl			11:00	21:30
			0.01 ng/µl			21:15	ND
			1 pg/µl			20:00	19:30
			0.1 pg/µl			37:30	ND
						ND	
	60991 a	*D. pauciseptata*	1 ng/µl			17:30	14:45
			0.1 ng/µl			20:15	21:30
			0.01 ng/µl			33:30	ND
						ND	
			1 pg/µl			42:15	ND
						ND	
			0.1 pg/µl			37:45	ND
						ND	
	60985	*D. anthuriicola*	1 ng/µl			16:15	18:15
			0.1 ng/µl			21:15	ND
			0.01 ng/µl			36:00	ND
						ND	
			1 pg/µl			46:15	ND
						ND	

			0.1 pg/µl	42:15	ND
				ND	
	n/a	RNase-free	0 ng/µl	33:45	ND
		water		ND	
	61352 c	*D.*	1 ng/µl	8:45	11:30
		macrodidyma	0.1 ng/µl	14:00	16:45
			0.01 ng/µl	38:00	ND
				ND	
			1 pg/µl	36:30	ND
				ND	
			0.1 pg/µl	32:45	ND
				ND	
	61263 f	*D. vitis*	1 ng/µl	14:15	14:15
			0.1 ng/µl	24:30	23:15
			0.01 ng/µl	30:30	ND
				ND	
			1 pg/µl	31:15	ND
				ND	
			0.1 pg/µl	34:30	ND
				ND	
	53498 a	*Ilyonectria* sp.	1 ng/µl	34:15	n/a
				ND	
	n/a	RNase-free	0 ng/µl	33:00	ND
		water		ND	

ND not detected or non-detection as indicated by detection after 30 minutes.