Development of a nested quantitative real time PCR for detecting *Phytophthora cinnamomi* in *Persea americana* rootstocks

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Abstract

Phytophthora cinnamomi causes Phytophthora root rot (PRR) in avocado (*Persea americana*), an important disease that causes severe economic losses to the avocado industry globally. To date, no PRR-resistant avocado rootstock variety has been discovered, although certain rootstock varieties have been shown to be more tolerant than others. In this study we developed an accurate, low cost assay for *in planta* quantification of *P. cinnamomi* to evaluate disease tolerance. A nested real time PCR assay was developed to sensitively detect pathogen DNA in plant tissues. Root samples from a highly tolerant (Dusa®) and less tolerant (R0.12) rootstock were collected at 0, 3, 7, 14 and 21 days after inoculation with *P. cinnamomi* and used for pathogen quantification. Nested primers developed in this study were specific and sensitive and could detect *P. cinnamomi* in root tissues. The amount of *P. cinnamomi* quantified in roots was significantly higher in the less tolerant R0.12 plants when compared to the highly tolerant Dusa® plants at all time points. This study has confirmed the known status of disease tolerance of Dusa® and R0.12 avocado rootstocks in a quantitative manner and provides a reliable molecular tool to

assist with industry breeding programs for the selection of PRR-resistant avocado rootstock varieties.

Introduction

Phytophthora root rot (PRR) is an important disease of avocado caused by the oomycete *Phytophthora cinnamomi* Rands. It has resulted in severe crop losses in most avocado producing countries around the globe. The first symptoms are brown to black brittle roots which develop after a brief latent period. Thus far pathogen quantification of *P. cinnamomi* in woody hosts such as avocado has not yet been attempted.

Traditionally, *P. cinnamomi* detection is based on the microscopic identification of the pathogen after culturing on selective media. However, pathogen quantification by these methods is not entirely reliable as calculations of pathogen biomass by microscopy are laborious and results can differ greatly between investigators (12). Chemical methods such as fatty acid ergosterol and carbohydrate chitin are used to determine the amount of a specific bio-molecule either present within pathogen cells or released into the environment (5, 17). Although widely implemented and not as laborious as microscopy, these methods lack specificity and become problematic when field samples are processed and required sample size is high.

In recent years, several techniques have been developed to enhance the detection of *P. cinnamomi* (4). Although conventional PCR has been useful for numerous *Phytophthora* species, it has not been successful when low levels of target DNA as with latent infections, are encountered. Most importantly, conventional PCR is not quantitative. In contrast quantitative real time PCR (qPCR) allows fast, reliable and accurate detection and quantification of plant pathogens (9,11). Sensitivity of qPCR can be greatly enhanced by implementing a nested approach in which a first round of amplification is carried out with conventional PCR and the

resulting product is then quantified in a second step by real time PCR. With nested PCR two primer pairs are designed based on the sequence of a target gene, one of which is nested within the other. Specificity of real time and real time nested PCR can be assessed by gel electrophoresis, melting curves and by sequencing (11).

The degree of pathogen colonization within a plant may correlate with resistance or susceptibility to a pathogen. In such cases, real time PCR is an ideal tool for detecting differences in host resistance or susceptibility. Qi and Yang (13) were able to show that resistance to rice blast, caused by *Magnoporthe grisea*, could be accurately evaluated with real time PCR analysis for the pathogen. By the time lesion development became visible, *M. grisea* was 80 times higher in a susceptible, compared to a resistant rice cultivar. Another study on alfalfa indicated significant correlations between the amount of *Phytophthora medicaginis* and the severity of disease it caused in this host (16). We developed a nested quantitative PCR method for *in planta* quantification of *P. cinnamomi* in two avocado rootstocks displaying different levels of tolerance to PRR. This assay confirmed the known phenotypic tolerance levels of available avocado rootstocks to PRR and therefore provides a molecular tool that can be used in avocado breeding programs to stream-line and fast-track the selection of rootstocks with high levels of PRR tolerance in a quantitative manner.

Materials and Methods

***Phytophthora* isolates and plant materials.** The *P. cinnamomi* isolate used for infection assays was provided by Westfalia Technological Services (WTS), situated in Tzaneen, Limpopo, South Africa. In addition, primer specificity was assessed against 21 *Phytophthora* spp. and 12 *P. cinnamomi* isolates selected from different geographical locations (Table 1). Nine-month-old avocado plants of two commercial rootstocks, Dusa® (highly tolerant) and R0.12 (less tolerant),

were also provided by WTS for *in planta* experiments.

Preparation of zoospore suspension. *Phytophthora cinnamomi* was first grown on V8 agar plates (50 ml of filtrated V8 juice, 0.5 g CaCO3, 20 g agar, distilled water is added to make up one liter, autoclaved at 121°C for 15 min) for five days. Small agar blocks (10 x 5 mm) containing mycelia were cut from the actively growing margin of the plates and transferred onto empty 90-cm-diameter Petri plates, to which 25 ml of 2% V8 broth (20 ml filtrated V8 juice, 0.2 g $CaCO_3$ in one liter dH_2O) was added and incubated for three days at room temperature (ca. 25°C). The broth was removed and agar blocks containing mycelia were rinsed three times with sterilized distilled water after which 25 ml of Whatman 1 mm-filtered stream water was added to each plate. Plates were incubated for two to three days at room temperature under UV light. Sporangia formation was monitored during this incubation period and once sufficient mature sporangia were observed, the plates were cold shocked by incubating at 4°C for 45 min after which they were removed and left at room temperature for one hour to stimulate zoospore release. The zoospore suspension was decanted from the plates, pooled together and used for inoculation.

Inoculation and sample collection. Avocado roots of two rootstocks (Dusa®and R0.12) were submerged for an hour in a five liter container containing zoospore suspension at a concentration of 7.2 x 10^4/ml (mock inoculated plants were submerged in sterile water) after which they were transplanted into 1.5 liter plastic bags filled with perlite (Chemisphere technologies, Gauteng, South Africa). Once transplanted, the zoospore suspension that was used to infect was divided into even portions and added to treated plants (50 ml per plant). Root tissue was collected at 0, 3, 7, 14 and 21 days post infection (dpi). Root material from three plants per rootstock was harvested per time point, snap-frozen in liquid nitrogen, ground to a fine powder

with a homogenizer (IKA A11 Basic analytical mill, United Scientific (Pty) Ltd., San Diego, USA) and stored at – 80°C. The experiment was repeated. **DNA extractions from mycelia and plant tissues.** DNA from pure *Phytophthora* cultures was extracted by using PrepMan™ Ultra Reagent (Applied Biosystems, Foster City, California, USA). Mycelia (ca. 50 mg) were placed in a 1.5 ml eppendorf tube together with 100 µl of PrepMan™ Ultra Reagent. The tubes were heated for 5 min at 95°C, where after the mycelia were homogenized in the tube by using a micro pestle and further incubated for 5 min at 95°C, followed by centrifugation for 10 min at 10 000 rpm. Supernatant was collected and diluted five times with sterile water and kept at -20 °C until further use. The concentration of all DNA samples was determined using a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, Inc., Montchanin, USA). DNA from infected avocado root material was extracted following the method described by Brunner *et al.* (1).

Primer design and pathogen quantification. The amount of plant genomic DNA present within each sample was quantified by real time PCR using primers amplifying a portion of the avocado *Actin* gene. The primers Actin-fwd (5'-GTATTCATTCACCACTACTG-3') and Actin-rev (5'- AGTCAAGAGCCACATAAG-3') were designed based on a *Persea americana* Actin sequence (GenBank accession number GU272027). A normal one step real time PCR was used for the plant *Actin* gene. The amount of plant DNA was calculated based on a standard curve constructed from different known amounts of avocado genomic DNA.

The amount of *P. cinnamomi* DNA present within samples was quantified using a nested real time PCR approach. This helps to increase the sensitivity of the assay since the detection of pathogen DNA in the early stage of infection can be problematic due to the low concentrations of pathogen DNA. Attempts to use YPh1-fwd *(5'*- CGACCATKGGTGTGGACTTT-3') and YPh1-rev (5'-ACGTTCTCMCAGGCGTATCT-3') as outer primers and Ycin-fwd (5'-

GTCCTATTCGCCTGTTGGAA-3') and Ycin-rev (5'-GGTTTTCTCTACATAACCATCCTATAA -3') as inner primers developed by Schena *et al.* (15) did not yield reliable results in this study. Therefore we selected *Lpv*, which encodes putative storage proteins in zoospores of *P. cinnamomi*, as the target gene for pathogen quantification (10). Primers for the first round PCR (outer PCR) were LPV3-fwd (5'-GTGCAGACTGTCGATGTG-3') and LPV3-rev (5'-GTGCAGACTGTCGATGTG-3') as developed by Kong *et al*. (8). Primers for the second round nested PCR, LPV3N-fwd (5'-GTGCAGACTGTCGATGTG-3') and LPV3N-rev (5'-GAGGTGAAGGCTGTTGAG-3'), were designed to bind within the outer PCR product based on the alignment of LPV3 genes from several *P. cinnamomi* isolates infecting avocado and eucalyptus. The outer PCR was carried out as a conventional PCR with only 15 cycles, using the LPV3-fwd and LPV3-rev primers. The second real time nested PCR was carried out using primers LPV3N-fwd and LPV3N-rev, with the outer PCR product as template. The amount of pathogen DNA was calculated based on a standard curve constructed from different known amounts of *P. cinnamomi* DNA.

PCR protocols. *Conventional PCR* (as for LPV3 outer PCR). PCR reactions were carried out in a total volume of 25 µl containing 2.5 µl 10X PCR reaction buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 0.2 µM of each specific primer, 1 U Fast Start *Taq* DNA polymerase (Roche Applied Science, Mannheim, Germany) and 20 to 50 ng of template DNA. Amplification conditions were as follows: 95°C for 5 min, followed by 15 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final elongation step at 72°C for 10 min. Amplifications were performed in an Eppendorf Master Cycler® gradient (Eppendorf, Hamburg, Germany).

Quantitative real-time PCR. Quantitative real-time PCR was performed using the Bio-rad® CFX 96 instrument (Bioline Ltd., London, England). A 20-µl reaction for PCR

amplification contained 10 µl Sensimix^{TM} SYBR No-ROX (Bioline Ltd., London, England), 0.25 µM of each of the forward and reverse primer, 2 µl template (2 µl of first round PCR product was used as template in the case of LPV3) and 3 µl PCR grade water. Thermal cycling conditions for actin were as follows: pre-incubation for 10 min at 95°C (hot start) followed by 40 cycles, each consisting of 15 s denaturing at 95°C, 15 s annealing at 60°C and 15 s primer extension at 72°C. Thermal cycling conditions for LPV3N were: pre-incubation for 10 min at 95°C (hot start) followed by 40 cycles, each consisting of 5 s denaturing at 95°C, 5 s annealing at 60°C and 5 s primer extension at 72°C. Negative controls contained water as template. All PCR reactions were performed in triplicate on each of the three biological replicates of two separate experiments. Melting curves were acquired at the end of the PCR run over the range of 65 to 95°C, increasing the temperature stepwise by 0.5°C every 5 s to confirm that individual q-PCR signals corresponded to a single homogenous DNA product. For assessment of PCR success and specificity, PCR products were separated by electrophoresis using 2% agarose gels, stained with GelRed (Biotium, Inc., California, USA) and visualized under UV light.

Statistical analysis. Standard regression curves were calculated from amplification data from the serial dilutions as follows: $y = mx + b$, where b = y-intercept of standard curve line (crossing point) and m = slope of the standard curve line (function of PCR efficiency) (6). All statistical analyses were conducted with the Statistics Online Computational Resource (SOCR) software (Los Angeles, CA). A Shapiro-Wilk normality test was performed prior to Mann-Whitney unpaired t-test.

Table 2.PCR primers used in this study, their target genes and product information.

Primer name	Sequence (5′ → 3′)	Target gene	Product size	Reference
LPV3-for	GTGCAGACTGTCGATGTG	*Lpv3*	450	Kong *et al.* 2003
LPV3-rev	GAACCACAACAGGCACGT	*Lpv3*		Kong *et al.* 2003
LPV3N-for	GTCACGACCATGTTGTTG	*Lpv3*	77	This study
LPV3N-rev	GAGGTGAAGGCTGTTGAG	*Lpv3*		This study
Actin-for	GTATTCATTCACCACTACTG	*Actin*	77	This study
Actin-rev	AGTCAAGAGCCACATAAG	*Actin*		This study

Results

Primer design. Real time primer pairs designed for actin and LPV3N consistently amplified the expected single bands of 77 bp for both products (Table 2). No cross amplification was observed when actin primers were tested on *P. cinnamomi* DNA or when LPV3N primers were tested on *P. americana* DNA.

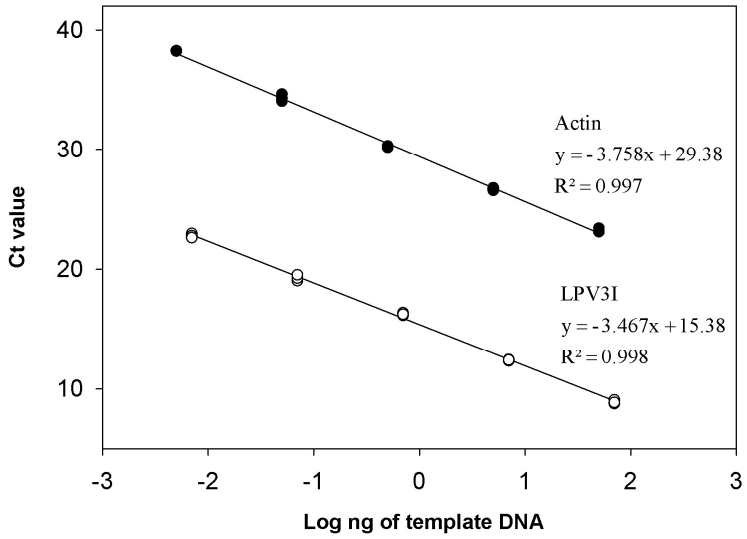

Fig. 1. Standard regression curve plots to assess the sensitivity of the qPCR assay. A dilution series of avocado and *Phytophthora cinnamomi* DNA spanning five orders of magnitude (1:0, 1:10, 1:100, 1:1000, 1:10000) amplified with Actin and LPV3N was used to generate standard curves for each separate primer pair.

PCR efficiencies and linearity. Known concentrations of ten-fold serially diluted DNA from *P. cinnamomi* and *P. americana* were used to construct standard curves (Fig. 1). Primer pairs showed high qPCR efficiency rates with high linearity (Fig. 1). Standard curves indicated consistent amplification over the different concentrations of template DNA used.

Sensitivity and specificity. To determine the sensitivity of the assay, a conventional and nested PCR was performed using different amounts of *P. cinnamomi* DNA, ranging from 200 ng/µl to 2 ag/µl. The lowest concentration detected using conventional PCR was 20 pg (Fig. 2). When nested PCR was used, the detection limit was decreased to 20 fg, a 1000-fold enhancement of detection sensitivity.

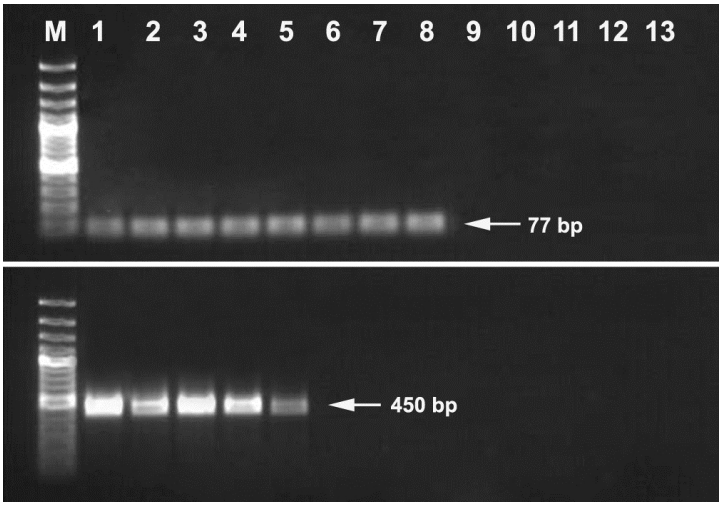

Fig. 2. Top -Sensitivity analysis of nested PCR for *Phythophthora cinnamomi*. The first-round PCR was performed using LPV3 as primer pair, and indicated amounts of *P. cinnamomi* DNA as template. The second-round PCR was performed using LPV3N as primer pair and amplified products from first-round PCR was used as template. Amplification products were analyzed by gel electrophoresis. Lane M : 100 bp ladder; 1: 200 ng; 2: 20 ng; 3: 2 ng; 4: 200 pg; 5: 20 pg; 6: 2 pg; 7: 200 fg; 8: 20 fg; 9: 2 fg; 20: 200 ag; 11: water control. Bottom -Sensitivity analysis of conventional PCR for *Phythophtora cinnamomi*. Amplification products were analyzed by gel electrophoresis. Lane M : 100 bp ladder; 1: 200 ng; 2: 20 ng; 3: 2 ng; 4: 200 pg; 5: 20 pg; 6: 2 pg; 7: 200 fg; 8: 20 fg; 9: 2 fg; 20: 200 ag; 11: water control.

The specificity of the assay was tested with DNA samples from 21 *Phytophthora* spp. (one to two isolates per species were tested) by PCR using LPV3N-fwd and LPV3N-rev primers. The results showed that LPV3N-fwd and LPV3N-rev primers were specific for *P. cinnamomi* and only produced a fragment of 77 bp for all *P. cinnamomi* isolates. No cross-amplification was observed with other *Phytophthora* spp. (Table 1). Additionally, melting curve analysis of real time PCR products resulted in single dissociation peaks with specific melting temperatures for LPV3N (at 82°C) and actin (at 77°C) confirming that the primers were specific for their target sequences (Fig. 3).

***In planta* monitoring of *P. cinnamomi*.** The growth of *P. cinnamomi* was assessed for three weeks in inoculated avocado rootstocks by qPCR. Total genomic DNA was extracted from infected root materials which contained both plant and pathogen DNA. The amount of plant DNA was quantified by using primers (Actin-fwd and Actin-rev) specific for the avocado *actin* gene. For quantification of *P. cinnamomi* DNA, primers (LPV3-fwd, LVP3-rev and LPV3N-fwd and LPV3N-rev) were used in a nested real time PCR. Pathogen load was determined by comparing the amount of pathogen DNA to the amount of plant DNA for each individual sample.

The quantitative nested PCR was sensitive enough to detect pathogen DNA at all time points except for non-inoculated control (0 dpi). The trend of the ratio of *P. cinnamomi* DNA per plant DNA over the different time points was the same for the highly tolerant rootstock (Dusa®) and the less tolerant rootstock (R0.12), however, the amount of *P. cinnamomi* DNA was significantly higher in R0.12 at all time points ($P<0.001$) (Fig. 4). In both the highly tolerant (Dusa®) and less tolerant (R0.12) rootstocks the amount of *P. cinnamomi* DNA increased over a

Table 1. Isolates of *Phytophthora* species used in this study to confirm the specificity of the nested primers designed.

Species	Host	Location	CMW	PCR result Amplified by LPV3N
Phytopthora cinnamomi	*Persea americana*	South Africa, Limpopo, Tzaneen		+
Phytopthora cinnamomi	*Lupinus angustifolius*	Australia, NSW, Caboolture	29596	+
Phytopthora cinnamomi	*Lupinus angustifolius*	Australia, Queensland, Caboolture	29597	+
Phytopthora cinnamomi	*Eucalyptus gummifera*	Australia, NSW, Kioloa	29606	+
Phytopthora cinnamomi	*Unknown*	Australia, NSW, Murwilumbah	29608	+
Phytopthora cinnamomi	*Persea americana*	South Africa, Limpopo, Tzaneen		+
Phytopthora cinnamomi	*Persea americana*	South Africa, Limpopo, Tzaneen		+
Phytopthora cinnamomi	*Persea americana*	South Africa, Limpopo, Tzaneen		+
Phytopthora cinnamomi	*Persea americana*	South Africa, Limpopo, Tzaneen		+
Phytopthora cinnamomi	*Persea americana*	South Africa, Kwazulu-Natal, Howick		+
Phytopthora cinnamomi	*Persea americana*	South Africa, Kwazulu-Natal, Howick		+
Phytopthora cinnamomi	*Persea americana*	South Africa, Kwazulu-Natal, Howick		+
Phytopthora cinnamomi	*Persea americana*	South Africa, Kwazulu-Natal, Howick		+
Phytophthora alticola	*Eucalyptus bajensis*	South Africa, Kwazulu-Natal, Midi Illovo	26295	–
Phytophthora alticola	*Eucalyptus bajensis*	South Africa, Kwazulu-Natal, Paulpietersburg	26296	–
Phytophthora arecae	Unknown	South Africa, Western Cape, Stellenbosch	19436	–
Phytophthora arecae	Unknown	South Africa, Western Cape, Stellenbosch	19437	–
Phytophthora boehmeriae	*Eucalyptus smithii*	South Africa, Kwazulu-Natal, Howick	19440	–
Phytophthora boehmeriae	*Eucalyptus smithii*	South Africa, Kwazulu-Natal, Ixopo	19439	–
Phytophthora cactorum	*Cussoniapaniculata*	South Africa, Northern Cape, Krugersdorp	1259	–
Phytophthora cactorum	apple seedling rootstock	South Africa, Western Cape, Grabouw	1260	–
Phytophthora citricola	Lucerne	South Africa, Northern Cape, Kimberley	1264	–
Phytophthora citricola	Lemonshoots / eureka	South Africa, Western Cape, Paarl	1265	–

Species	Host	Location	CMW	PCR result Amplified by LPV3N
Phytophthora citrophthora	Citrus rootstock	South Africa, Limpopo, Letaba Estate	20206	–
Phytophthora citrophthora	Citrus rootstock	South Africa, Western Cape	20204	–
Phytophthora colocasiae	-	Unknown	20201	–
Phytophthora colocasiae	Protea	South Africa, Western Cape	22018	–
Phytophthora crytogea	*Vitisvinifera*	South Africa, Western Cape	19411	–
Phytophthora crytogea	Pinussp	South Africa, Western Cape	19410	–
Phytophthora drechsleri	*Solanumtuberosum*	Argentina, Cordoba	28869	–
Phytophthora drechsleri	*Beta vulgaris*	USA, California	28870	–
Phytophthora eucalypti spnov	Eucalyptus sp	South Africa, Kwazulu-Natal, Ingwe	22024	–
Phytophthora eucalypti spnov	*Eucalyptus smithii*	South Africa, Kwazulu-Natal	22029	–
Phytophthora foliorum	Azalea	USA, Tennessee	31064	–
Phytophthora frigida	*Eucalyptus smithii*	South Africa, Kwazulu-Natal, Lion River	19433	–
Phytophthora frigida	*Eucalyptus smithii*	South Africa, Kwazulu-Natal, Pietermaritzburg	19434	–
Phytophthora humicola	Soil from citrus orchards	Taiwan, Changhua, Yungching	28866	–
Phytophthora humicola	Citrus	Taiwan	28867	–
Phytophthora inundata	*Aesculus Hippocampus*	UK, Buckinghamshire, Claydon	29595	–
Phytophthora megasperma	*Medicagosativa*	Canada, Ontario, Dundas County	28865	–
Phytophthora megasperma	*Medicagosativa*	Canada, Ontario, Dundas County	28864	–
Phytophthoramultivora	Soil	South Africa, KZN, Umtamvuna Nature Reserve, Port Edward	35209	–
Phytophthoramultivora	Soil	South Africa, KZN, Umtamvuna Nature Reserve, Port Edward	35210	–
Phytophthora nicotianae	Citrus sp	South Africa, Limpopo, Tzaneen	19442	–
Phytophthoranicotianae	*Acacia mearnsii*	South Africa, Kwazulu-Natal, Lion River	19443	–
Phytophthora palmivora	*Kentia Palm*	Australia, Queensland, Caboolture	29599	–
Phytophthora palmivora	*Arecastrumromanzoffianum*	Australia, Queensland, Pimpama	29601	–
Phytophthora parasitica	*Acacia mearnsii*	South Africa, Mpumalanga, Tygerkloof / Piet Retief	1521	–
Phytophthora parasitica	*Acacia mearnsii*	South Africa, Mpumalanga, Tygerkloof / Piet Retief	1522	–

Species	Host	Location	CMW	**PCR result** Amplified by LPV3N
Phytophthora pgchlamydo	river	South Africa, KZN, Ingeli forest, Weza	35258	–
Phytophthora pgchlamydo	river	South Africa, KZN, Ingeli forest, Weza	35257	–
Phytophthora quininea	*Cinchona officinalis*	Peru, Region of Tingo Maria	31061	–
Phytophthora quininea	*Cinchona officinalis*	Peru, Region of Tingo Maria	31062	–

CMW - Culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

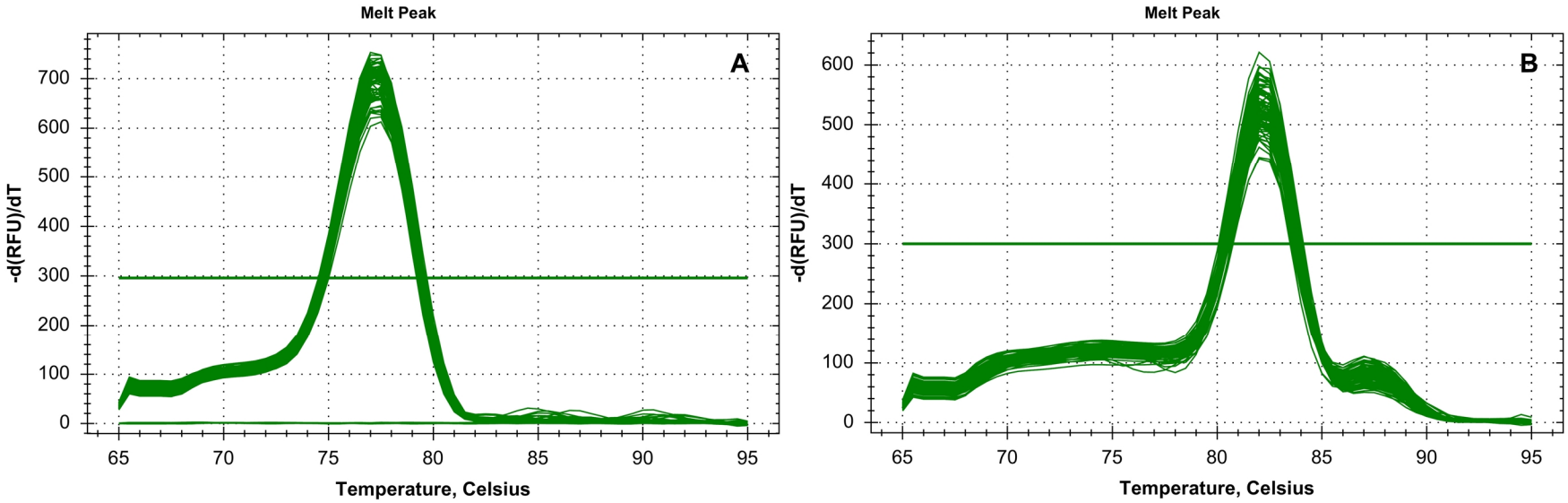

Fig. 3. Melting curve analysis for actin (A) and LPV3N (B) samples assayed by qPCR. The negative first derivative of the normalized fluorescence was plotted against the temperature to determine the melting temperature (T_m) of the amplicons generated during qPCR analysis.

seven day period post inoculation. Between seven and 21 days post inoculation the amount of *P. cinnamomi* DNA declined. However at 21 days there was detectable DNA in both rootstocks.

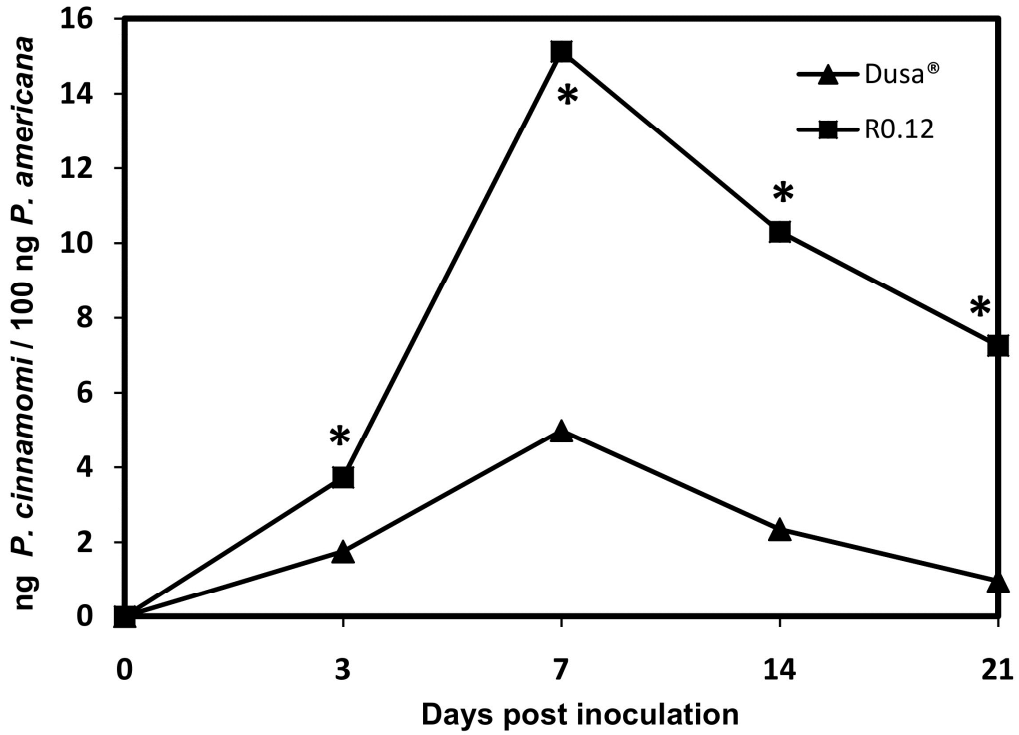

Fig. 4. Monitoring of *Phytophthora cinnamomi* growth by qPCR in inoculated avocado root tissues. Pathogen load was quantified from infected root tissues by normalizing the LPV3N values with the corresponding actin values for each individual sample. Data were analyzed with the SOCR software package using a Mann-Whitney unpaired t-test (at each time point, $P < 0.001$).

A significantly higher ratio of *P. cinnamomi* DNA to plant DNA in the susceptible R0.12 at each time point was present when compared to the highly tolerant Dusa®.Visual observations showed similar results. R0.12 roots had more necrotic lesions and were black and brittle compared to the tolerant Dusa® (Fig. 5).

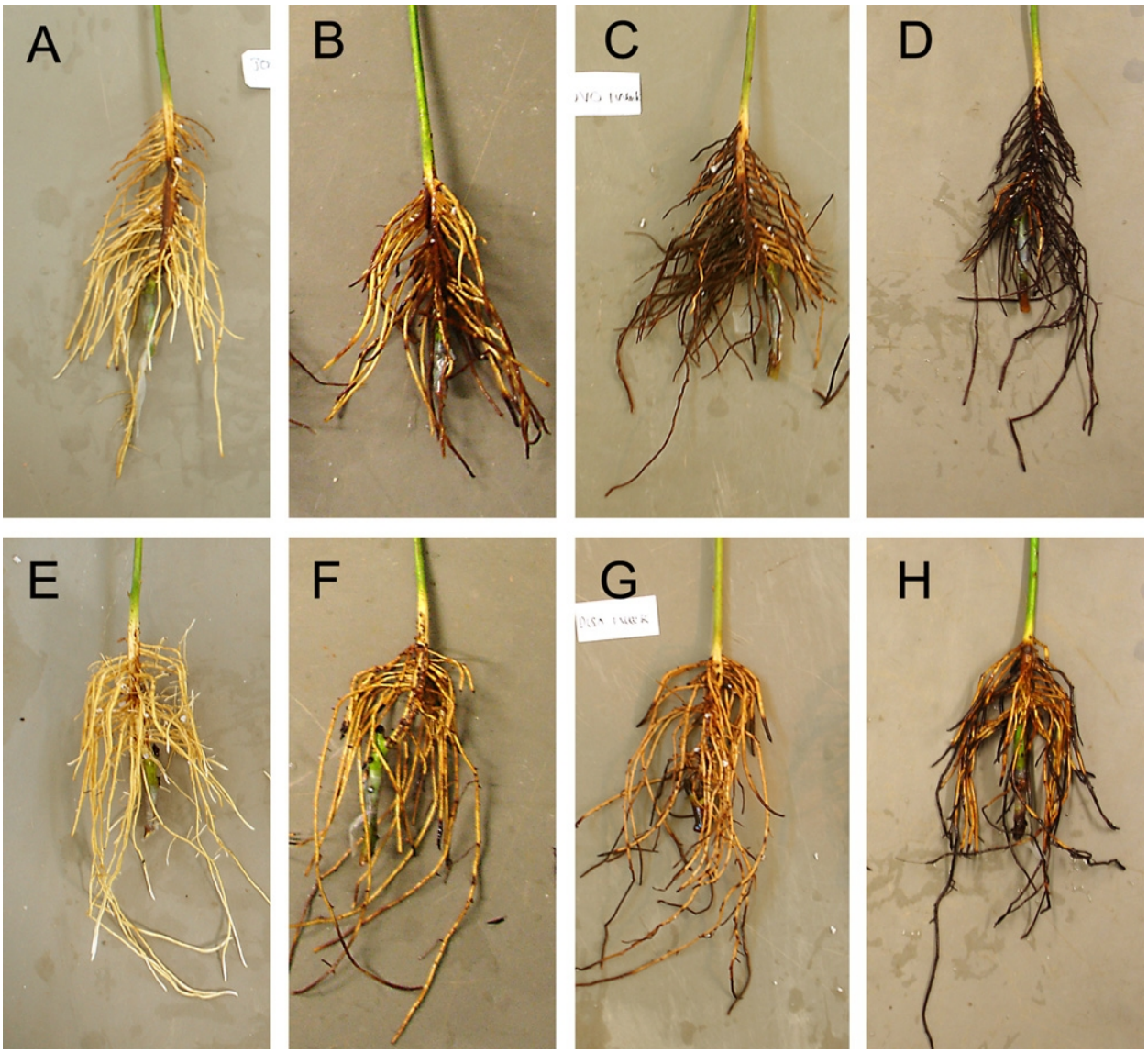

Fig. 5. Root symptoms on Dusa® (highly tolerant) and R0.12 (less tolerant) rootstocks after *Phytophthora cinnamomi* infection: R0.12 roots at 0 dpi (A), 3 dpi (B), 7 dpi (C), 14 dpi (D) and Dusa® roots at 0 dpi (E), 3 dpi (F), 7 dpi (G), 14 dpi (H).

Discussion

A nested real time PCR was developed that allowed specific, sensitive and quantitative detection of *P. cinnamomi* within root tissues of avocado. This assay was highly sensitive, with detection limits as low as 20 fg of *P. cinnamomi* DNA. This is an improvement in sensitivity vs other DNA based detection methods for *P. cinnamomi* where 100 fg could be detected (15).

Previously designed primers (LPV3-fwd and LPV3-rev) for the *Lpv3* gene by Kong *et al*. (8) successfully amplified a portion of the *Lpv3* gene from *P. cinnamomi* with a detection limit up to 20 pg of *P. cinnamomi* DNA. By designing a second pair of primers (LPV3N-fwd and LPV3N-rev), nested within the first PCR product and using these two primer pairs together in a nested PCR system, we were able to increase the sensitivity of the assay to detect as little as 20 fg of *P. cinnamomi* DNA. The *Lpv 3* gene, which encodes for a putative storage protein in the large peripheral vesicles in zoospores of *P. cinnamomi*, consists of 12 to 18 highly conserved 534 bp repeat units (10). Placing primers in this unique repetitive unit, improved the specificity and sensitivity of detection for this species. The nested primer pairs designed by Schena *et al.* (15), which were based on a single copy gene, the ras related protein gene *Ypt1*, did not amplify any product when DNA from infected avocado root tissue was used. However, since these primer pairs have only been used to detect *P. cinnamomi* DNA from pure cultures they may not be sufficiently sensitive for use with infected plant tissue (15).

In this study, the amount of pathogen DNA was directly normalized with the host plant DNA and therefore provided accurate and reliable results when compared to techniques that are based on detection of pathogen DNA only. The effect of varying amounts of starting material as well as any PCR inhibitor present in the sample was standardized for both plant and pathogen DNA. Moreover, the use of a plant *actin* gene also served as an internal control to eliminate false negative results. *Phytophthora cinnamomi* is known to be a hemi-biotroph; thus, one would expect plant DNA to be degraded when the infection becomes necrotrophic and this would give biased results towards pathogen DNA. For example, with the necrotic fungus *Botrytis cinerea* on grapes Diguta *et al*. (3) noted inaccuracy of pathogen quantification when normalizing to host DNA. The correlation between the amount of pathogen DNA in relation to plant DNA and the

susceptibility should be well maintained. This biased effect towards pathogen DNA in a necrotrophic system can be overcome by spiking foreign DNA in the extraction protocol to use it as a normalization parameter as has been shown in various studies (3,4).

The maximum amount of *P. cinnamomi* DNA in infected roots was observed at 7 dpi for both the highly tolerant (Dusa®) and less tolerant rootstock (R0.12). After 7 dpi, the ratio of pathogen DNA in relation to plant DNA was reduced. This could be due to the elimination of pathogen biomass in the dead tissue as well as to the generation of new roots from the plants.

In this study, the amount of *P. cinnamomi* DNA in avocado roots as determined by real time PCR correlated well with the known level of tolerance for these two rootstocks. The amount of *P. cinnamomi* DNA in roots of highly tolerant Dusa® was significantly lower ($P<0.001$) when compared to that of the less tolerant R0.12 at all time points. Pathogen DNA quantified in roots ranged from 3.73 to 15.12 ng per every 100 ng of plant DNA for R0.12 and 0.97 to 4.98 ng per every 100 ng of plant DNA for Dusa®, emphasizing the different tolerance levels of these rootstocks.

Dan *et al.* (2) used a PCR based procedure to differentiate between tolerance and resistance to *Verticillium dahliae* in potato and suggested that accurate quantification of pathogen biomass in potato should be measured and used as an indicator in breeding for resistance. The quantitative nature of real time PCR can be very useful in plant breeding programmes as it allows comparisons to be made between cultivars with different and even subtle degrees of tolerance or resistance. Our work has proven that this technique is a valuable tool for quantitative pathogen diagnosis as well as for monitoring colonization and disease development. Accurate quantitative measurements of pathogen colonization in host plants is also of great importance as it contributes to a better understanding of the interaction of avocado with

P. cinnamomi.

This study provides researchers and the industry with a valuable tool to quantify *P. cinnamomi in planta*. It allows quantification and comparison of the level of infection in avocado rootstocks with varying levels of tolerance. This is the first report that correlates the phenotypic tolerance observed in avocado rootstocks with molecular evidence. This assay has proven to be a useful molecular tool that could be used in breeding programmes where the screening for resistant or highly tolerant varieties against PRR could be speed up and applied in the screening for new anti-oomycete compounds.

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