Studying the interaction between *Phytophthora cinnamomi and Persea americana using gene expression profiling and pathogen quantification.*

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by

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Declaration

I, Juanita Engelbrecht, hereby declare that this dissertation, submitted to the University of Pretoria for the degree MSc Plant Pathology, contains my own work, and that the content contained within this thesis has not been submitted to any other university or institution.

Juanita Engelbrecht

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PREFACE

Avocado (*Persea americana* Mill.) is a popular and nutritious crop and of great importance to the global fruit industry. One of the major problems encountered is the susceptibility of avocado trees to Phytophthora root rot (PRR) caused by the soil-born oomycete *Phytophthora cinnamomi* Rands. Control is achieved through an integrated control strategy that consists of mulching, chemical control in the form of phosphite injections and the use of resistant rootstocks. Emphasis is now placed on the use of resistant rootstock varieties to combat this devastating disease. Avocado breeding programmes around the world have provided growers with a selection of highly tolerant rootstocks such as Duke 7 and Dusa®. Despite the availability of tolerant material it has not yet been established why certain rootstocks display high levels of tolerance against *P. cinnamomi* and others not. Selecting resistant rootstocks is a time consuming process that can take up to 25 years. Due to the lack of research on the avocado/*Phytophthora* interaction, the aim of this dissertation was i) to establish a pathogenicity system that could be used to study the interaction ii) to investigate the expression profiles of selected defense-associated genes from five avocado rootstocks upon *P. cinnamomi* infection and lastly to develop an assay that could detect *P. cinnamomi in planta* that could be applied to aid in the selection process of tolerant avocado varieties.

Chapter 1 entitled Plant defense mechanisms against *Phytophthora* provides an overview of plant defense responses against *Phytophthora* and where possible specifically against *P. cinnamomi.* General plant defense concepts including pathogen triggered immunity and specific effector triggered immunity is discussed. Both these immune responses are linked to other key players that regulate specific signalling pathways in order to achieve an effective defense response. Mechanisms involved in defense that are discussed include cell wall reinforcements, production of ion fluxes and ROS species, MAPK and phosphorylation cascades, rapid induction of defense genes, accumulation of defense-related proteins

including phytoalexins and PR proteins which all negatively affect colonization of potential pathogens.

Chapter 2 reports on the establishment of two reliable small plant inoculation systems for studying plant disease development in the greenhouse followed by subsequent molecular studies. Briefly a hydroponics system was evaluated to allow easy access to root material for RNA extractions followed by quantitative PCR and the second solid system was evaluated using perlite and vermiculite as growth substrates to assess disease development and severity in three avocado rootstocks with varying levels of resistance to PRR.

In Chapter 3 the role of seven defense related genes were investigated in five avocado rootstocks after infection with *P. cinnamomi*. The expression of each individual gene was assessed over seven time points ranging from 0 to 72 hours using quantitative RT-PCR. Data were analyzed statistically to highlight differences amongst the five rootstocks with respect to their gene expression against the pathogen.

Chapter 4 describes the development of a nested quantitative PCR that quantifies *P. cinnamomi in planta* in two avocado rootstocks displaying different levels of tolerance against this soil-borne oomycete. A nested primer set was developed for the *Lpv* gene that resulted in a *P. cinnamomi* – specific, sensitive assay that can be utilized to assess rootstock tolerance.

The thesis concludes with a discussion (Chapter 5) on all the data generated during the course of this study, our findings and recommendations.

LIST OF ABBREVIATIONS AND SYMBOLS:

%	Percent
˚C	Degree Celcius
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
ABA	Abscisic acid
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
ARFs	Auxin response factors
ATP	Adenosine triphosphate
AUX/IAA	Auxin/Indole-3-acetic acid
Avr	Avirulence
BAK1	BRI1-associated receptor kinase
bp	Base pair
BRs	Brassinosteroids
$CaCO_3$	Calcium carbonate
Ca^{2+}	calcium
cDNA	Complementary DNA
CDPKs	Calcium dependant protein kinases
Cl^-	Chloride
CLKs	Cytokinins
CTAB	Cetyltrimethylammoniumbromide
CWDE	Cell wall degrading enzymes
dH_2O	Distilled water
DNA	Dioxyribonucleic acid
dNTP	Deoxynucleotide Triphoshate
Dpi	Days post inoculation
EDTA	Ethylenediaminetetraacetic Acid
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
F3H	Flavanon-3-Hydroxylase
g	Gram
g/L	Gram per liter
Gas	Gibberellins
H_20	Water
H_2O_2	Hydrogen peroxide

HCl	Hydrochloric acid
hpi	Hours post infection
hr	Hours
HR	Hypersensitive response
H^+	Hydrogen
HRGP	Hydroxyproline-rich glycoproteins
IBA	Indolebutyric
IFS	Isoflavone synthase
JA	Jasmonic acid
K^+	Potassium
KCl	Potassium chloride
Kda	Kilo Dalton
L	Liter
LRR	Leucine rich repeat
M	Molar
MAPK	Mitogen activated protein kinase
MAPKKKs	MAPK kinase kinases
MAPKKs	MAPK kinases
mg	Milligram
min	Minutes
ml	Milliliter
mm	Millimeter
mM	Millimolar
NaCl	Sodium chloride
NARPH	hymexazol; ampicillin; rifampicin; pentachloronitrobenzene
NBS	Nucleotide binding site
NBS-LRR	Nucleotide binding site Leucine rich repeat
NCBI	National Center for Biotechnology Information
ng	Nanogram
NO	Nitric oxide
NPP1	Necrosis inducing Phytopthora protein 1
NPR1	Nonexpressor of PR-1
PAL	Phenylalanine ammonia lyase
PAMPs	Pathogen-associated molecular patterns
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PR	Pathogenesis related
PRR	Phytophthora root rot
PRR	Pattern recognition receptors
PTI	Pamp triggered immunity

qRT-PCR	Quantitative Real-time PCR
R-genes	Resistance genes
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	ribosomal RNA
RT	Reverse transcriptase
RXLR	ARG-X-LEU-ARG
SA	Salicylic acid
SAR	Systemic acquired resistance
sec	Seconds
Spp	Species
TMV	Tobacco mosaic virus
U	Units
u/µl	Units per microlitre
v	Volume
VIGS	Virus induced gene silencing
W	Weight
WTS	Westfalia Technological Services

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CHAPTER 1

Plant defense mechanisms against *Phytophthora* spp.

INTRODUCTION

Plants like other organisms, must survive numerous challenges they encounter in their environments. One of these challenges is that plants have to defend themselves against a vast range of pathogens such as bacteria, fungi, oomycetes, viruses and nematodes. Most plants are capable of defending themselves by using a variety of defense mechanisms. However, little is known about what proportion of a plant genome is allocated for defense. This would include all genes that are involved in the recognition of the pathogen, downstream signalling events, as well as genes involved in the response to counter the pathogen. Although defense responses have been studied in numerous plants, relatively little information is available in comparison with the vast amount of unexploited host-pathogen interactions. Moreover, defense mechanisms vary between different plant species in response to specific pathogens, therefore each particular pathosystem of interest should be studied individually.

Species of *Phytophthora* belong to one of the most economically important groups of plant pathogens in the world. This group has caused significant damage to the forestry and agricultural crop industry. The most famous example is *Phytophthora infestans* (Mont.) de Bary that caused global devastation in 1845 during the Irish potato famine (De Bary, 1876). The recent outbreak of *Phytophthora ramorum* Werres, De Cock & Man in`t Veld, causing extensive mortality of *Quercus* spp. and *Lithocarpus densiflorus* in California and Oregon is yet another example illustrating the impact these species can have on natural forests (Davidson *et al.*, 2003; Rizzo *et al.*, 2002). In terms of forest plantations, the recently described *Phytophthora pinifolia* Alv. Durán, Gryzenh. & M.J. Wingf. is associated with a serious needle disease of *Pinus radiata* in Chile, causing severe economic losses (Durán *et al.*, 2008). There are many other examples of diseases caused by *Phytophthora* spp. worldwide (Erwin & Ribeiro, 1996).

Of the many important diseases occurring on fruit crops, Phytophthora root rot (PRR) of avocados (*Persea Americana* Mill.) is caused by the soil-borne pathogen, *Phytophthora cinnamomi* Rands which is commonly known as the avocado root rot or cinnamon fungus (Zentmyer, 1980). The pathogen causes significant damage on the African continent and is highlighted as one of the most damaging diseases in South African avocado orchards, where it has been shown to reduce crop yields drastically. Its occurrence is worldwide and places like California and Australia have had severe economic losses (Allen *et al.*, 1980; Coit, 1928). Primary infection of *P. cinnamomi* on avocado occurs at the absorbing feeder roots, resulting in a brownish- to black brittle appearance. There is almost no progression into the larger roots (Zentmyer, 1980). In the advanced stages of infection, small feeder roots may be completely absent, making it impossible for the trees to obtain enough water and nutrition (Pegg, 1991). Although the disease has been studied for more than 60 years not a single definite control strategy has been implemented, and annual losses continue to increase.

Currently, phosphite trunk injections are the main method of preventing and controlling PRR on avocados, as well as several other hosts. As part of integrated disease management, resistant rootstocks are used in conjunction with phosphite injections, since the use of resistant rootstocks is considered the best method for controlling PRR. The tolerance/resistance observed in certain rootstocks can be explained by the rapid regeneration of active feeder roots, while in others the progress of infection is restricted in the roots, either biochemically or structurally (Sánchez-Pérez *et al.*, 2008). Unfortunately, the moderate resistance of commercially available rootstocks itself is not sufficient to ensure effective disease control under environmental conditions that are favourable for root rot. It is also noteworthy of mention that reduced sensitivity against phosphite was reported by Dobrowolski *et al.* (2008) in avocado orchards after prolonged use of phosphonate, and therefore much attention is now given to the use of alternative control measures and improvement and selection of resistant rootstock varieties.

Defense responses to plant pathogens are becoming the main focus of many intensive research activities, given that current technologies offer the possibility of genetically engineering plants for broad-based effective resistance against pathogens. Studying plant pathogen interactions is of great importance, not only to contribute to our understanding of basic plant defense processes, but also to improve agricultural productivity. Resistance genes (*R*-genes) are prime candidates for increasing resistance by means of conventional breeding, molecular breeding and transgenic strategies (McDowell & Woffenden, 2003). They are detected and identified much easier when compared to polygenic resistance. There are many potential *R*-genes in defense responses of plants that can be utilized for beneficial interest. Some of the most well known resistance mechanisms against *Phytophthora* spp. include structural features of the host, preformed chemical inhibitors, induced structural barriers as well as the hypersensitive response (HR) (Moy *et al.*, 2004). Polygenic resistance is almost more powerful than *R*-gene resistance as it is known to give moderate resistance to various micro-organisms that cannot be easily overcome as is the case with *R*-gene resistance. This makes it more valuable and durable for breeding programmes. Polygenic resistance can be introduced into plant varieties by making use of quantitative trait loci analysis as well as molecular markers.

Phytophthora cinnamomi is a major pathogen of avocado, and desperate measures need to be taken in order to combat yield losses caused by this pathogen. Knowledge on the genetic basis of defense responses would greatly contribute to the understanding of these complex defense mechanisms as well as in the application of plant breeding and disease control practises. However, due to a lack of knowledge, intensive research is required on this topic.

This review will therefore shed light on the defense response of various host plants against *Phytophthora* spp. with special emphasis on *P. cinnamomi* where possible.

GENETIC BASIS OF PLANT DEFENSE STRATEGIES

Plant pathogen interactions

Plants are capable of surviving many extreme and versatile challenges, despite the constant threat of developing new diseases from potential pathogenic organisms (Jackson & Taylor, 1996). Plant pathogens can obtain nutrients by three major lifestyles: necro-, bio- or hemi-biotrophy. A necrotrophic pathogen actively kills plant tissues as it colonizes, obtaining nutrients from dead cells. A biotrophic pathogen obtains nutrients from living plant cells (Agrios, 2007), whereas a hemibiotroph exhibits both necro- and biotrophic phases during disease development. Oomycetes can be necro-, bio- or hemi-biotrophic, of which *P. cinnamomi* is a hemi-biotroph (Hardham, 2007; Jackson & Taylor, 1996).

During evolution, plants have developed effective mechanisms to counteract pathogen invasion. There are three scenarios that could cause a pathogen's attempt to invade and spread in plant tissues to be unsuccessful. Firstly, pathogens fail to establish when the plant is not able to support the niche requirement of the particular pathogen. Secondly, the plant may possess preformed structural barriers and biochemical compounds that restrict the invading pathogen. Thirdly, when a pathogen gains entry into the plant and the plant is able to recognize the pathogen, defense mechanisms are activated that restrict the pathogen from colonizing (Hammond-Kosack & Jones, 1996). If a pathogen is not able to cause disease in the first two scenarios, the plant is considered to be a non-host species whereas in the case when a pathogen successfully overcomes all three scenarios and is able to cause disease, the host plant is considered to be a susceptible host species. The term 'susceptibility' refers to an interaction between a susceptible plant and a virulent pathogen resulting in disease; also referred to as a compatible interaction. An incompatible interaction, on the other hand, refers to an interaction between a resistant plant and an avirulent pathogen resulting in no disease (Agrios, 2007).

Recognitioning

Recognitioning is one of the first events that occur in any plant-pathogen interaction. At an organism level, recognitioning can be interpreted as the response of an organism to a substance produced by another organism, i.e. a signal. Therefore one could assume that the recognizing organism plays an active role, whereas the recognized organism would undertake a passive role (Tyler, 2002). Recognitioning includes a series of events by which pathogens such as *P. cinnamomi* perceive the presence of plant tissue and then adjust their physiology so that they are capable of infecting the specific host tissue encountered (Tyler, 2002). In order to counter the defense responses activated by the plant, pathogens adapt to utilize the nutrients acquired from the plant tissue.

Recognitioning can be divided into recognition of the host by the pathogen and recognition of the pathogen by the host. Limited research is available explaining the complicated series of signal exchange that occurs specifically during the infection process. Recognition of the host by the pathogen includes detection of chemical, electrical, and physical properties of the specific host tissue encountered (Tyler, 2002). Recognition of the pathogen by the host involves recognizing substances or structures of the pathogen such as elicitors and cell wall fragments that have been shown to trigger defense responses in the host plant. Such substances are referred to as pathogen associated molecular patterns (PAMPs) and causes a basal defense response called PAMP triggered immunity (PTI) (Zipfel & Felix, 2005).

Biochemical and genetic approaches have been used to discover pathogen and host signals in *Phytophthora* pathosystems. Biochemical approaches have identified specific pathogen-derived compounds such as elicitors that have been shown to be involved in the activation of defense responses in host and non-host species (Tyler, 2002). Receptors of certain elicitors have also been identified. Genetic approaches have aided in the discovery of avirulence and resistance genes that interact in a gene-for-gene way.

Plant immunity

Plants, like animals, also use two distinct levels of defense to recognize and respond to pathogen attack. PAMP triggered immunity is an evolutionary more ancient primary response, and is based upon the recognition of microbial structures termed PAMPs by pattern recognition receptors (PRR) (Fig. 1, 2) (Ausubel, 2005; Chisholm *et al.*, 2006; Jones & Dangl, 2006; Zipfel & Felix, 2005). PAMP triggered immunity constitutes a range of universal responses, including ion fluxes, mitogen activated protein kinase (MAPK) cascades, production of reactive oxygen species (ROS), cell wall reinforcements and rapid induction of defense genes largely regulated by WRKY transcription factors (Ingle *et al.*, 2006). Other responses such as the production of callose and the accumulation of defense-related proteins including chitinases and glucanases also form part of PTI (Van Loon *et al.*, 2006b).

PAMP triggered immunity is effective against non-adaptive pathogens and is also called non-host resistance or basal resistance. PAMPs have been defined as surface-exposed, abundant structures that are common to microbial sources. They are not found in potential eukaryotic hosts and are necessary for microbial lifestyle (Medzhitov & Janeway, 1997). Oomycetes secrete several proteins that can act as PAMPs, such as elicitins, transglutaminase, cellulose-binding proteins and cell wall derived components such as *β*-glucan (Daxberger *et al.*, 2007; Kamoun, 2006). PAMPs act outside the plasma membrane (Gijzen & Nurnberger, 2006). Many oomycete PAMPs and other elicitors are secreted proteins which activate defense responses in the host plant (Van Loon *et al.*, 2006b). One of the first molecules to be considered a PAMP was Pep 13, which is a 13 amino acid peptide motif of a *Phytophthora sojae*-derived cell-wall transglutaminase (Nürnberger *et al.*, 1994). This motif is one of the most conserved sequences shared among many species of *Phytophthora*.

Pattern recognition receptors recognize a particular domain of a larger PAMP molecule that often possesses structural enzymatic functions that are crucial for the pathogen's behaviour or survival. Thus far, no plant genes have been cloned that encode for PRRs specifically against oomycete PAMPs (Stassen & Van den Ackerveken, 2011). All known PRRs in plants are plasma membrane occupant proteins and thus allow for recognition at the cell surface (He *et al.*, 2007). Effective pathogens are able to evade or suppress PTI by secreting effector molecules/proteins that act outside or inside the host cell to suppress or otherwise manipulate plant innate immunity (Hein *et al.*, 2009). This is called effector-triggered susceptibility (ETS). Effector triggered susceptibility represents the first level at which molecular co-evolution between a host and pathogen occurs. Pathogen effectors have been shown to suppress immunity via direct molecular interactions with host defense-associated proteins (Block *et al.*, 2008; Chisholm *et al.*, 2006). Two broad classes of effectors can be identified: those that act extra-cellularly to suppress secreted defense-related proteins; and those that act intra-cellularly, presumably to suppress the defense-related signal transduction, regulation and trafficking that leads to PTI or effector triggered immunity (ETI) (Hein *et al.*, 2009). It has been demonstrated that defense responses of both PTI and ETI show significant overlap (Tsuda *et al.*, 2008).

Biotrophic fungi and oomycetes have the ability to deliver effector proteins into the host cell by making use of haustoria (Fig. 3). Haustoria are specialized structures that form within plant cells, which remain enclosed in an adapted plant cell membrane, known as the extrahaustorial membrane (Hahn & Mendgen, 2001; Panstruga, 2003). Evidence exists that haustoria play a critical role in the secretion of cytoplasmic fungal and oomycete effectors into the plant cell (Catanzariti *et al.*, 2006; Dodds *et al.*, 2004; Whisson *et al.*, 2007). Oomycetes like *P. infestans*, are also known to secrete apoplastic effectors apart from the cytoplasmic effectors (Damasceno *et al.*, 2008; Kamoun, 2006). Oomycete effectors contain a distinctive internal motif Arg-X-Leu-Arg (RXLR) that might be necessary for their delivery into plant cells. Numerous studies have shown that several oomycete RXLR effectors are

able to suppress host immunity. Expression of avirulence proteins in host cytoplasm often leads to *R*-gene dependent cell death. One of the methods to suppress PTI responses is by *Agrobacterium* mediated transient expression of effector genes. Co-expression of the *P. infestans Avr3a* was able to inhibit hypersensitive cell death caused by a *P. infestans* protein, INF1 elicitin, indicating a potential virulence function (Bos *et al.*, 2006). This indicates that oomycete RXLR effectors often function in suppression of plant immunity. Recognition occurs inside the plant cell and these avirulence (Avr) proteins are delivered across plant cell walls during infection (Dodds *et al.*, 2009).

To defend themselves against pathogens, plants have developed a secondary defense response that is triggered upon recognition of effectors or effector-mediated molecules of host targets (Chisholm *et al.*, 2006; Jones & Dangl, 2006). Effectors that enable pathogens to overcome PTI are recognized by intracellular receptors that are products of specific *R*-genes from the classically defined gene-for-gene system. These receptors that recognize plant effectors activate other defense mechanisms. This secondary defense is known as ETI and involves direct and indirect recognition of effectors by various R proteins. If one effector is recognized by a corresponding nucleotide binding site leucine-rich repeat (NBS-LRR) protein (usually encoded by *R*-genes), this will result in ETI. Most effector molecules are products of *Avr* genes. Effectors recognised by R proteins are termed Avr proteins, although the term ‘avirulence’ is limiting in that the same protein with an avirulence function in an incompatible interaction may possess a virulence function in a compatible interaction. For this reason the term ‘effector’ is more widely accepted. The final outcome of the combat between the plant and the pathogen depends on the ability of the pathogen to suppress the plant’s defense system and the ability of the plant to recognize the pathogen and initiate further defense responses. Current progress in the study of biotrophic oomycetes indicated that this group follows a very similar response as the overall model proposed for pathogen effector/host immunity interactions (Birch *et al.*, 2008; Ellis *et al.*, 2007; Tyler, 2009).

Plants are also protected by a mechanism called systemic acquired resistance (SAR) which occurs at sites distant from PTI and ETI that protects undamaged plant tissue from subsequent pathogen attack. This occurs by means of PTI- and ETI-mediated pathogen recognition (Grant & Lamb, 2006). Systemic acquired resistance is effective against a broad range of pathogens and is dependent on different plant hormones, including salicylic acid (SA), jasmonic acid (JA), ethylene (Et) and abscisic acid (ABA), or combinations thereof (Glazebrook, 2005; Grant & Lamb, 2006; Thomma *et al.*, 2001). Durrant & Dong (2004) showed that transgenic potato plants expressing the bacterial salicylate hydroxylase gene (*nahC*), were not capable of expressing SAR when lower leaves were treated with arachidonic acid prior to infection with *P. infestans.* This led to the conclusion that SA is a very important and essential intermediate in the SAR signalling pathway (Durrant & Dong, 2004).

Systemic acquired resistance is associated with increased levels of SA as well as the activation of *PR* genes as it was observed that increased levels of PR proteins accompanied the heightened resistance observed in many plant-pathogen interactions. However it was also discovered that in cases of over expression of *PR* genes that it did not always have a additive effect on resistance emphasizing the involvement of other mechanisms other than *PR* genes (Vernooij *et al.*, 1994). *Nonexpressor of PR gene 1* (*NPR1*) came forth as a vital component of SA signalling. *Nonexpressor of PR-1* acts as a transcriptional co-activator of *PR* gene expression (Mishina & Zeier, 2007). Given its important roles in plant immunity, SAR is currently one of the most valuable mechanisms in plant defense research.

Effector triggered immunity (ETI)

Gene-for-gene mediated resistance is now referred to as ETI. Because effector-R protein interactions are highly specific, ETI in plants appears to be very similar to adaptive immunity in animals. Effector triggered immunity is expressed through the same responses as PTI, although it is a much faster and stronger response (Fig. 1, 2) (Truman *et al.*, 2006). Effector

triggered immunity often results in the HR (Greenberg & Yao, 2004), and is associated with additional locally induced defense responses that block further growth of the pathogen.

The traditional view of *R*-gene perception by an *Avr* gene, was based on the gene-for-gene concept, which suggested a direct interaction between both protein products (Flor, 1971), similar to the receptor ligand relationship (Fig. 4). According to this model, an *Avr* gene of a pathogen encodes an elicitor protein that is recognized by a receptor protein. This receptor protein is encoded by a matching *R*-gene in the host plant (Keen, 1990). In the majority of cases, however, recognition of an Avr protein by a cognate R protein may be indirect, involving the activations of the R protein on recognition of effector activity on virulence targets. This is also known as pathogen induced modified self molecular patterns, previously described in the guard hypothesis (Dangl & Jones, 2001; Van Der Biezen & Jones, 1998) which states that pathogens are detected indirectly through the action of their effectors (Jia *et al.*, 2000). The guard hypothesis supports the view that R proteins function as sensors to guard the targets of the pathogen effector molecules, hence the guardee protein (Fig. 4). The gaurdee protein specializes in perception of the effector by the NBS-LRR protein. When R proteins sense interference, resistance is activated. Both of these models proved to be correct, however, in different plant pathosystems. New data on indirect effector recognition has emerged that seems not to fit within any of these two models, therefore a new decoy model has been proposed. The decoy model takes evolutionary aspects of selection forces of the guardee protein into account (van der Hoorn & Kamoun, 2008).

The number of R proteins and matching Avr proteins that have been identified in plant and pathogens respectively is increasing. During the last 10 years, numerous *R*-genes and *Avr* genes have been cloned from many plant species (Dangl & Jones, 2001; Hammond-Kosack & Parker, 2003). Although only a few plant resistance proteins have been characterized, evidence is suggesting that plants use both direct and indirect mechanisms of pathogen recognition and thus direct or indirect detection of effectors by R proteins. Effectors and R

proteins are fast-evolving - reflecting an evolutionary battle between the pathogen and the host.

Resistance genes encode at least five diverse classes of R proteins (Chisholm *et al.*, 2006). In addition to the NBS-LRR class, these include cytoplasmic signal-transducing serine-threonine kinases, extracellular LRRs with transmembrane anchors, extracellular LRRs with transmembrane receptors and cytoplasmic serine threonine kinases. The largest class of R proteins involved in recognition events possess NBS and LRR domains. Although ample information has been gathered through extensive research, exactly how effector recognition leads to NBS-LRR activation still remains unclear. The NBS-LRR domain is conserved and is thought to transform direct and indirect signals to activate defense responses (Collier & Moffett, 2009). It is also suggested that signal activation may involve an exchange of Adenosine triphosphate (ATP) and Adenosine diphosphate (ADP) in the binding site (Tameling *et al.*, 2006). Current models postulate that many of these NBS-LRR classes form multiprotein interactions which can maintain R proteins in their inactive form until effectors are detected (Friedman & Baker, 2007). The LRR domain might play a dual role, acting both as a recognition determinant and as a repressor that prevents inappropriate NBS activation (Belkhadir *et al.*, 2004). Many structural and functional domains and regions have been identified that can interact in various combinations to form different R proteins, but little is known about their immediate roles. The LRR domain appears to play a central role in protein-protein interactions by regulating recognition specificity - particularly in effector-protein binding (DeYoung & Innes, 2006; Kobe & Deisenhofer, 1994; Rairdan & Moffett, 2006), whereas the NBS region has a critical role through either nucleotide binding or hydrolysis (Martin *et al.*, 2003).

Some oomycete *Avr* genes that have been identified are *Avr1b-1*, *Avr1a* and *Avr3*a from *Phytophthora sojae (*Kaufmann and Gerdeman) (Qutob *et al.*, 2009; Shan *et al.*, 2004) *Avr3a, Avr4*, and *Avr-blb1* from *P. infestans* (Armstrong *et al.*, 2005; Van Poppel *et al.*, 2008;

Vleeshouwers *et al.*, 2008). Examples where *R*-genes have been discovered include the *R3* locus of potato *(Solanum tuberosum L.)* that confers full resistance to avirulent isolates of *P. infestans* (Huang *et al.*, 2004) as well as two other late blight *R*-genes that have been cloned from *Solanum demissum* Lindl, *R1* (Ballvora *et al.*, 2002) and *R3a* (Huang *et al.*, 2005). These *R*-genes all encode NBS-LRR type R proteins that are predicted to reside in the cytoplasm.

Currently, there is little known about how exactly defense mechanisms mediate resistance. It is well known that defense in plants is initiated by *R*-gene recognition and that it is regulated by various signalling pathways, however, the exact details are still unknown - especially with reference to the avocado-*Phytophthora* interaction as there in no gene-for-gene interaction and resistance is thought to be polygenic. Perhaps due to the large amount of gene expression changes that occur during PTI and ETI activation, the key changes responsible for the prevention of pathogen growth are not easy to identify. The possibility also exists that the responses observed might have such a minute effect that it is very difficult to pinpoint to an individual contribution / event.

STRUCTURAL DEFENSE MECHANISMS IN PLANTS

Cell wall fortifications

One of the first barriers that microbes encounter is the cell wall of plants. In order to reach the nutrient rich cytoplasm they need to move through this barrier. *Phytophthora cinnamomi* undergoes a necrotrophic phase, and actively degrades the cell wall, and therefore strengthening of the cell wall could lower the efficiency of degrading enzymes and consequently prevent the toxins from entering the cytoplasm (Hammond-Kosack & Jones, 1996). Physical barriers such as the cell wall, cuticle and epicular waxes successfully prevent infection by non-pathogenic oomycetes (Attard *et al.*, 2008). Evidence exists for an accumulation of cell wall fortification compounds, such as glycoproteins, lignin, suberin and

callose at the point of entry of the pathogen, as well as in cells surrounding the area undergoing HR (Benhamou & Nicole, 1999; Hammond-Kosack & Jones, 1996).

Apart from the role of hydroxyproline-rich glycoproteins (HRGP) in cell wall strengthening, cross-linking of these proteins in cell walls has been shown to increase resistance against pathogen-derived cell wall-degrading enzymes (CWDE) (Hückelhoven, 2007). A more direct role of these glycoproteins against pathogens has also been identified. In tobacco, an HRGP of 12 kDa agglutinated zoospores of compatible and incompatible races of *Phytophthora parasitica* var. *nicotianae* B. de Haan (Mellon & Helgeson, 1982). Protein cross linking by means of basic HRGPs within the cell walls also plays a key role in the organization of the primary cell wall design and may act as a central point for the induction of lignin and suberin polymerization (Showalter, 1993).

Both lignin and suberin fulfill various roles during plant growth and development and act as cell wall fortification compounds - especially during plant defense against pathogens (Franke *et al.*, 2002). Lignin and suberin form a physical barrier that prevents penetration by an invading pathogen and also reduces water loss from plant cells (Moerschbacher *et al.*, 1990). Lignins are complex aromatic heteropolymers, composed mainly of three phenylpropanoid-derived hydroxyl-cinnamyl alcohol monomers, namely p-coumaryl, coniferyl, and sinapyl alcohols (Boerjan *et al.*, 2003). Evidence for the fundamental role of lignification in resistance has previously been demonstrated in the incompatible interaction between tobacco (*Nicotiana tabacum* L.) and *P. infestans.* Tobacco leaves infiltrated with INF1 elicitors showed that lignin content increased slightly at 12 hours and reached a peak that was 1.5 times more when compared to the control where leaves were infiltrated with Tris buffer (Wang, 2004).

One of the structural responses observed after pathogen attack is the rapid deposition of callose surrounding the point of entry. Callose deposition by plants is a means of cell wall fortification to prevent further penetration of the pathogen. Callose is a heterogeneous β-1,3-

glucan that is deposited between the cell wall and the plasma membrane and is used as a classical marker of PTI responses after treatment with PAMPs or non-infectious pathogens. Callose deposition was recently shown to depend on PAMP-induced glucosinolates (Clay *et al.*, 2009). It acts as a permeability barrier and strengthening or sealing agent during growth and development (Stone & Clarke, 1992). Although the order of events in PTI is still not clear, callose deposition may be downstream of ROS production (Nicaise *et al.*, 2009). Callose may act as a substance in which the toxic compounds excreted by the pathogen can be deposited; it may also slow down nutrient transfer from plant cells to the pathogen, or hinder pathogen growth for long enough until other plant defenses can be induced (Stone & Clarke, 1992). Judith *et al*. (2005) conducted a study where leaves of *tobacco* were infected with *Phytophthora nicotianae* B. de Haan*. At 1 hour post infection (hpi) callose deposition could already be observed at the infection site.* Initially, callose distinctively appeared only at the joining of mesophyll cells, but later callose depositions occurred across the whole cell wall of infected cells. It was also discovered that almost all the cells producing H_2O_2 also exhibited callose depositioning (Judith *et al.*, 2005).

Another type of cell wall fortification that occurs rapidly in response to microbial invasion is the formation of papillae. Papillae are often formed immediately beneath the penetration peg and are heterogeneous in composition (Heath, 1980). They are thought to physically block pathogen penetration in host cells (Bayles *et al.*, 1990). Hächler and Hohl (1984) conducted a time course study investigating the development and distribution of collars and papillae in tuber tissues of resistant (Eba) and susceptible (Bintje) potato cultivars after inoculation with *P. infestans.* They found that there were eight times more papillae in the resistant cultivar when compared to the susceptible cultivar relative to the total number of wall appositions (Hächler & Hohl, 1984). In another study the formation of papillae and callose were observed between the cell wall and plasmalemma in seven field resistant plant species, which included eucaluptus, acacias, cereals and sedges after being inoculated with *P. cinnamomi.*

Callose was also found to be deposited in dense hemispherical pads next to hyphal penetration structures as well as intercellular hyphae (Weste & Marks, 1987).

BIOCHEMICAL DEFENSE MECHANISMS IN PLANTS

Although structural defense has been shown to provide some level of defense against attacking pathogens in certain cases, it is clear that resistance of plants against a pathogen does not rely so much on its structural barriers as on the substances produced in the cell before or after infection. This is evident from examples where a pathogen is not capable of infecting certain plant varieties where no structural barrier of any kind seems to be present or have developed. Plants are thus able to use chemical defense either as pre-formed or as induced mechanisms (VanEtten *et al.*, 1994).

Signal transduction

Inducible defenses must be activated as soon as possible after the pathogen has been detected in order for resistance to be achieved. Therefore it is very important that signal transduction and regulation processes must restrict the distribution of inducible defenses to the correct place and at the correct time (McDowell & Dangl, 2000). Despite the major advances that have been made towards our understanding of the molecular mechanisms of recognition in pathogen-specific resistance, the precise signalling pathways that initiate and manage defense mechanisms still remain unclear. Most of the problems encountered when attempting to understand signalling, results from the complexity of signalling cascades and crosstalk between common pathways in response to various stimuli (Genoud & Métraux, 1999). Despite all of these problems, quite a few important signalling components have been identified such as ion fluxes, ROS, specific protein intermediates and phosphorylation cascades that will be discussed briefly.

Ion fluxes

One of the earliest responses to PAMPs is a rapid change in ion fluxes across the plant plasma membrane. Changes in ion fluxes can occur within 10 minutes after recognition which leads to membrane depolarisation and extracellular alkalinisation as well as an increase in cytosolic calcium (Boller & Felix, 2009; Garcia-Brugger *et al.*, 2006; Nürnberger *et al.*, 1994). Influxes of both hydrogen (H^+) and calcium (Ca^{2+}) as well as effluxes of potassium (K^+) and chloride (Cl^-) have been observed after treatment with PAMPs (Garcia-Brugger *et al.*, 2006; Jabs *et al.*, 1997; Pugin *et al.*, 1997). Ion fluxes are believed to be a consequence of H^+-ATPases, and plasma membrane-bound ion-channels that are activated by dephosphorylation (Atkinson & Baker, 1989). Calcium signalling in response to plant-pathogenic extracts (including phytotoxins and elicitors) has been reported across a wide range of species (Jabs *et al.*, 1997; Lecourieux *et al.*, 2002). GP 42 that has been narrowed down to Pep13, was one of the first PAMPs to be identified which has been shown to stimulate PTI responses such as ion fluxes and the production of ROS species in parsley cells infected with *P. sojae* (Brunner *et al.*, 2002).

Calcium acts as an important second messenger and could be the golden key involved in the activation of other PAMP responses (Zipfel & Robatzek, 2010). Calcium not only amplifies signals perceived from cell surface receptors but also passes these signals on to target molecules which in turn can activate other responses such as the HR (Balagué *et al.*, 2003; Hann & Rathjen, 2007; Heath, 2000; Jabs *et al.*, 1997; Lecourieux *et al.*, 2006). Pharmacological studies on tobacco cells treated with cryptogein, an elicitin from *Phytophthora cryptogea* Pethybr. & Laff, have shown that an influx of Ca^{2+} was required for various defense signalling events (Lecourieux *et al.*, 2006; Tavernier *et al.*, 1995). In many plant pathogen interactions a biphasic increase in cytosolic Ca^{2+} can be observed, for example in *Arabidopsis* plants inoculated with *Pseudomonas syringae* pv. *tomato*, the initial

Ca^{2+} increase is induced in an *R*-gene-independent manner followed by a second burst, which requires a functional *R*-gene product (Grant *et al.*, 2000).

Nemchinov *et al*. (2008) proposed a time schedule for Ca^{2+} ion fluxes in the mediation of plant defense responses; this model was based on their data obtained from studies conducted on the *Nicotianca benthamiana* / *P. syringae* patho-system. They suggested that the first temporary Ca^{2+} influx across the plasma membrane occurred within the first 10 minutes as a result of PAMPs recognition by host receptors, as described by Grant *et al*. (2000). This phase was said to activate basal defense responses. This was followed by a 'silent' Ca^{2+} response 10 – 60 min after challenge that may reflect a shift period from PTI to the continuous *R*-gene-dependent calcium increase, similar to a proposal made by Grant *et al*. (2000). Prolonged Ca^{2+} uptake, which can occur up to seven hours after the challenge, indicated that the pathogen overcame the initial PTI (Jones & Dangl, 2006). Sustained increases in Ca^{2+} at this stage were critical for the formation of ROS, the oxidative burst and induction of the HR (Grant *et al*. 2000). Finally after the HR pathway has been activated, Ca^{2+} levels declined to the last HR phase which is an expanded cell death phase. This indicated that Ca^{2+} signalling plays a role in both PTI and ETI defense responses. It seems that initial Ca^{2+} entry into the cells is required for the induction of basal defense responses and activation of the HR, while the late Ca^{2+} efflux could potentially reflect its role in the development of a more expanded cell death (Nemchinov *et al.*, 2008).

Reactive oxygen species (ROS)

The oxidative burst is one of the most rapid defense responses after pathogen recognition and gives rise to ROS within a few minutes; in particular superoxide (O_2^-), hydrogen peroxide (H_2O_2) and nitric oxide (NO) (Bhattacharjee, 2005). PAMPs are capable of triggering an oxidative burst (Chisholm *et al.*, 2006). Defense responses associated with the production of ROS include direct killing of pathogens, activation of host cell death (HR), accumulation of phytoalexins and increased cell wall strengthening (Bolwell & Daudi, 2009; Torres, 2010).

Recent studies have emphasized that ROS play a very important role in signalling of both PTI and ETI (Torres *et al.*, 2006; Van Breusegem *et al.*, 2008). However, the exact occurrence of signalling events via the oxidative burst during PTI still remains vague (Nicaise *et al.*, 2009).

There are many advantages of ROS as signalling molecules. Firstly the cell is capable of rapidly producing and scavenging different forms of ROS at the same time, thus enabling quick and active changes in ROS levels (Mittler *et al.*, 2011). Another advantage is the tight control over the sub-cellular localization of ROS signals within plant cells. Reactive oxygen species can also be used as long distance signals that can spread rapidly throughout the plant. Each cell along the path of the signal would be capable of activating its own ROS producing mechanism while transporting a ROS signal over long distances in the plant (Mittler *et al.*, 2011).

There are many different forms of ROS and each of them has their own unique molecular properties. Superoxide for instance is not capable of transferring passively across a membrane but can be dismutated without any difficulty into H_2O_2 that can easily move across membranes passively or through water channels (Lamb & Dixon, 1997; Miller *et al.*, 2007). Superoxide is a highly reactive unstable molecule that is formed under basal and light stress conditions (Triantaphylidès & Havaux, 2009). Hydrogen peroxide is the most stable ROS and its permeability across membranes is due to an unpaired electron (Yamasaki *et al.*, 1997). Superoxide and H_2O_2 can also mediate the formation of lipid peroxides that are membrane soluble. Reactive oxygen species could serve as a link to several other signalling pathways, their association with calcium and protein phosphorylation networks supports this statement. Doke (1983) was the first to report that superoxide anions were produced rapidly and transformed into H_2O_2 in the incompatible interaction between potato and *P. infestans*. Potato tuber protoplasts were treated with hyphal cell wall components. Their results

indicated that O_2^- generation and NADPH oxidation occurred on the plasma membrane of potato upon the recognition of host cells that lead to the HR (Doke, 1983).

Several enzymes have been implicated in the generation of ROS. Based on genetic analysis, the NADPH oxidase (also known as respiratory burst oxidases homologs or Rbohs) appears to be the most important enzymatic mechanism responsible for the production of superoxide radicals during the oxidative burst to defend cells against pathogen intrusion (Sumimoto, 2008; Torres *et al.*, 2006). Plant Rbohs can be found in many species (Torres & Dangl, 2005) where they localize to the plasma membrane consistent with their function in producing the apoplastic oxidative burst (Keller *et al.*, 1998; Kobayashi *et al.*, 2006; Sagi & Fluhr, 2001). Yoshioka *et al*. (2003) have isolated two *rboh* genes from *Nicotiana benthamiana* leaves. Virus-induced gene silencing (VIGS) indicated that both these *rboh* genes are required for H_2O_2 accumulation and for resistance againts *P. infestans*. *Nbrboh*-silenced *N. benthamiana* plants showed an increase in susceptibility and a reduced HR when treated with the avirulent *P. infestans* (Yoshioka *et al.*, 2003). Leborgne-Caste *et al.* (2008) showed that tobacco cells treated with a *Phytophthora* elicitor, cryptogein, stimulated clathrin mediated endocytosis and that this reaction produced ROS via *NtrbohD*. Although numerous studies have shown that the Rboh-NADPH oxidases are responsible for the production of ROS in response to pathogens, the outcome of disease resistance and cell death also relies on the specific plant–pathogen interaction (Torres, 2010).

Research has shown that avirulent pathogens are capable of inducing a biphasic ROS production in plants which consists of a low amplitude first phase followed by a much more prominent and sustained accumulation during the second phase (Lamb & Dixon, 1997; Torres *et al.*, 2006). The result is that when the second phase of ROS is induced the plant is able to recognise and respond thus preventing disease. Virulent pathogens that avoid host recognition induce only the first short lived, low amplitude phase of this response, emphasizing the role of ROS in the establishment of plant defense (Torres *et al.*, 2006).

When there is no second phase, the plant is not able to recognise the pathogen and this will result in disease development. The presence or absence of the second phase seems to play an important signalling role in the activation of plant defense.

Nitric oxide has also been identified as a potential signalling molecule in *R*-gene mediated resistance. Based on studies of *Arabidopsis* plants, Ali *et al*. (2007) proposed that PAMPs are recognised by unknown receptors which in turn trigger ion channels specifically Ca^{2+}, causing an increase in cytosolic Ca^{2+} that eventually results in the generation of NO (Ali *et al.*, 2007). Nitric oxide plays an important role in the HR, in that it diffuses to adjacent cells and then plays a role as a signalling molecule that activates other calcium mediated channels (Shetty *et al.*, 2008). Inhibitors of NO synthesis compromise *Arabidopsis* pathogen-specific resistance to *P. syringae* (Delledonne *et al.*, 1998) whereas addition of NO donors induce phytoalexin production and transcription of defense related genes in potato treated with *P. infestans* (Noritake *et al.*, 1996).

Phosphorylation cascades

The role of signal transduction through phosphorylation is crucial for plant resistance. Protein phosphorylation occurs in various cellular processes and is very important in regulating protein activity. Calcium-dependent protein kinases and mitogen-activated protein kinase (MAPK) have been identified for specific phosphorylation events in *R*-gene mediated resistance (Zhang & Liu, 2001). Signalling via the MAPK cascades makes use of directional and consecutive phosphorylation events. Mitogen-activated protein kinase networks seem to link upstream receptors to downstream targets, and are regarded as key components involved in the regulation of transcriptional changes that occur in induced cells (Hamel *et al.*, 2006). Many plant MAPK cascade pathways have been connected to various cellular processes which include responses to environmental stimuli such as pathogen infection, wounding and ROS (Colcombet & Hirt, 2008; Pitzschke *et al.*, 2009). The availability of genome sequences allows the identification of genes that could possibly play a role in MAPK

signalling cascades although only a few MAPK cascades have positively been linked to play an active role in plant defense signalling (Pitzschke *et al.*, 2009).

In a typical MAPK cascade pathway, excited membrane receptors activate MAPK kinase kinases (MAPKKKs) and become phosphorylated in one of two ways, either by the receptor itself or an interconnecting upstream protein kinase (Chang & Karin, 2001). Directional and consecutive phosphorylation continues as MAPKKK activates downstream MAP kinase kinases (MAPKKs) that in turn activate MAPKs. Mitogen-activated protein kinases then target various effector proteins in the cytoplasm or nucleus that include other kinases, enzymes, or transcription factors (Khokhlatchev *et al.*, 1998). Some of the most well known MAPKs are MPK3, MPK4 and MPK6. All of these have shown to be activated by a diverse range of stimuli including abiotic stresses, pathogens and oxidative stress. It is known that MPK4 negatively regulates biotic stress signalling whereas MPK3 and MPK6 act as positive intermediates of defense responses (Pitzschke *et al.*, 2009). The interchange between the positive regulation by MPK3/6 and the negative regulation through MPK4 seems to enable stringent control of defense responses to either advance or limit pathogen growth that ultimately results in plant death or remaining alive.

Calcium dependent protein kinases (CDPKs) are a large family of Ca^{2+}-binding proteins that function as Ca^{2+} sensors downstream from the calcium signal. Their participation in plant–pathogen interactions has been extensively studied. It has been proposed that CDPKs are capable of regulating NADPH oxidase in plant defense responses and mediate ROS production. It was reported that two potato (*Solanum tuberosum*) CDPKs could regulate the production of ROS during plant pathogen signalling through the phosphorylation of NADPH oxidase (Kobayashi *et al.*, 2007). A further study found that NADPH oxidase was regulated by Ca^{2+} (Sagi & Fluhr, 2001). Several CDPKs have been shown to operate as universal transcriptional activators during PAMP response signalling (Boudsocq *et al.*, 2010). In tobacco, ROS induced by cryptogein treatment requires the orthologous gene *NtRbohD*

whereas both *NbRbohA* and *NbRbohB* are needed for ROS production after treatment with *P. infestans* hyphal cell wall extracts (Yoshioka *et al.*, 2003). This work provided genetic evidence for the involvement of a MAPK cascade in the regulation of *rboh* genes (Yoshioka *et al.*, 2003). These Rboh enzymes are regulated by both Ca^{2+} binding as well as by phosphorylation via CDPKs (Kobayashi *et al.*, 2007; Ogasawara *et al.*, 2008). By amplifying and transducing PAMP derived signals detected by membrane receptors, these signals eventually cause changes in gene expression, thus emphasizing that plant MAPKs and CDPKs play a vital role in the activation of other defense mechanisms in plants.

Plant hormones that regulate signal transduction during pathogen resistance

It is common knowledge that plant hormones govern many developmental and physiological processes in plants. Downstream responses to ETI and PTI are much easier to understand when signalling pathways are unravelled. Knowledge on the involvement of hormones in resistance, more specifically in signal transduction and regulation processes has accumulated over the past few years. The most crucial and well known regulators of adaptive plant defense responses are the SA and JA–ET hormone pathways (Bari & Jones, 2009; Pieterse *et al.*, 2009). These two pathways can act synergistically and antagonistically to some level. It is known that generally SA is more involved in defense responses associated with biotrophic pathogens whereas JA-ET is more involved with necrotrophic pathogens and insects although there have been exceptions (Glazebrook, 2005; Kessler & Baldwin, 2002).

More recently, ABA (Asselbergh *et al.*, 2008; Mauch-Mani & Mauch, 2005), auxins (Navarro *et al.*, 2006; Wang *et al.*, 2007), gibberellins (Navarro *et al.*, 2008), cytokinins (Siemens *et al.*, 2006; Walters & McRoberts, 2006) and brassinosteroids (Nakashita *et al.*, 2003; Shan *et al.*, 2008) also emerged as important regulators of signalling. In numerous cases, these hormones have been shown to interact either antagonistically or synergistically with the SA-JA-ET backbone of the signalling network, thereby altering the specific outcome of the

defense response (Fig. 5) (Campos *et al.*, 2009; de Torres-Zabala *et al.*, 2007; De Vleesschauwer *et al.*, 2010; Jiang *et al.*, 2010; Navarro *et al.*, 2008; Ton *et al.*, 2009; Wang *et al.*, 2007; Yasuda *et al.*, 2008). The involvement of all of these hormones in plant immunity gives a good indication that the control of processes like plant growth, development, reproduction and defense is more complex than anticipated and is interlinked in a multifaceted network of cross-talk between different hormone signalling pathways. Therefore plant hormones are very important in the regulation of these processes. This type of cross talk network is very powerful in that it allows plants to rapidly adapt to the biotic and abiotic factors they encounter and to make the most of their resources in a cost-efficient manner. It is thought that induced defense responses evolved to save energy under pathogen-free conditions, as resources are only utilized when defenses are activated (Walters & Heil, 2007). Crosstalk between hormone signalling pathways provides the plant with the potential that allows the plant to adapt its defense responses to the specific pathogen encountered (Bostock, 2005; Pieterse & Dicke, 2007). While more and more hormones are being identified that are involved in signalling, it is becoming evident that various post-translational protein modifications also play a role in signalling and add to the complexity of the signalling network (Spoel *et al.*, 2010).

Salicylic acid

Salicylic acid was only considered to be a major determinant in plant resistance after it was observed that SA levels increased at the site of pathogen infection and later also in distal plant parts (Malamy *et al.*, 1990). It is well established that SA plays a very important role in plant defense due to its involvement in the activation of defense responses against biotrophic and hemi-biotrophic pathogens as well as in the development of SAR (Grant & Lamb, 2006). Attack by hemi-biotrophs or biotrophic pathogens triggers the SA-dependent signalling pathway. When this pathway is activated SA levels rise, leading to the activation of SAR and various defense effector genes including pathogenesis-related proteins 1 (PR-1),

PR-2 and *PR-5* (Glazebrook, 2005). Accumulation of the defense protein PR-1 has been observed following increases in SA and is commonly used as an indicator of SA-dependent defense responses (Xie *et al.*, 1998; Yalpani *et al.*, 1991).

Mutant and transgenic plants that are impaired in SA signalling are incapable of developing SAR and do not show *PR* gene activation upon pathogen infection, which has indicated that SA is a critical regulator in the SAR signalling pathway (Dixon *et al.*, 2002). It seems unlikely that SA is itself the mobile signal responsible for SAR (Vernooij *et al.*, 1994). The regulatory protein NPR1 emerged as an important transducer of the SA signal upon activation by SA. NPR1 interacts with TGA transcription factors that are involved in the activation of *PR* genes (Dong, 2004). Scrutiny of the NPR1 protein that operates downstream of SA accumulation in *Arabidopsis* has highlighted the importance of SA and NPR1 in the HR and callose accumulation (Rate & Greenberg, 2001). NPR1 can also function downstream of JA and ET independently of SA and SA can function independently of NPR1, suggesting that there is another branch of the SA signalling pathway (Pieterse *et al.*, 1998; Rairdan & Delaney, 2002; Reuber *et al.*, 1998; Uquillas *et al.*, 2004). This indicates the complexity and interplay between hormones in the regulation of plant resistance to pathogens.

The importance of SA as a signalling compound was investigated in potato against *P. infestans (*Halim *et al*., 2007). Transgenic potato plants that are not capable of accumulating SA were used. Although measurement of the lesions did not show significant differences between the wild type and transgenic potatoes, real time PCR revealed a higher level of the pathogen in the transgenic plants when compared to the wild type. This indicated that SA is one of the core compounds necessary for basal defense of potato plants against *P. infestans* since the inability to accumulate SA might account for the increased susceptibility observed (Halim *et al.*, 2007). Another study addressing the importance of SA in signal transduction against hemi-biotrophs was carried out on Pep 13 in potato. Pep 13 has been linked to the activation of an oxidative burst, accumulation of SA and JA, as well as a HR-like cell death

(Halim *et al.*, 2004). The discovery that Pep 13 signalling in potato needs both SA and JA is in contrast to the assumption that SA and JA signalling pathways are antagonistic (Kunkel & Brooks, 2002). Transgenic potato plants that are impaired to accumulate SA due to the expression of a salicylate hydroxylase have been shown to be more susceptible to biotrophic and hemi-biotrophic pathogens. Infiltration of Pep 13 onto the leaves of these transgenic plants, make them incapable to accumulate H_2O_2 or elicit a HR-like cell death (Halim *et al.*, 2004). Also the ability of JA-deficient plants to accumulate SA when compared to wild-type plants indicated that SA operates separately or upstream of JA in mediating Pep 13-induced defense responses.

Jasmonic acid and Ethylene

Jasmonic acid and ET are usually associated with defense against necrotrophic pathogens and herbivorous insects. Although SA and JA/ET defense pathways are generally viewed to be mutually antagonistic, there are numerous examples where synergistic interactions have also occured (Beckers & Spoel, 2006; Kunkel & Brooks, 2002; Mur *et al.*, 2006; Schenk *et al.*, 2000). This emphasizes that the signalling network activated in plants depends on the pathogen and its specific mode of entry. These stresses trigger the elevation of endogenous JA or other jasmonates that induce the expression of specific jasmonate responsive genes to combat the stress encountered. Jasmonic acid is involved in numerous processes such as seed germination, root growth, tuber formation, tendril coiling, fruit ripening, leaf senescence and stomatal opening (Bari & Jones, 2009), however their roles in plant defense responses are very important and has therefore received a lot of attention.

Jasmonic acid is synthesized through the octadecanoid pathway and begins with the release of linolenic acid from the chloroplast membrane, a process thought to be catalyzed by phospholipase (Creelman & Mulpuri, 2002). Jasmonic acid signalling is not well understood, but involves the ubiquitin ligase SCF^{COI1} and the JA-conjugating enzyme JAR1 (Devoto & Turner, 2003). Ethylene and JA defense signalling converge on induction of the histone

deacetylase *HDA19* and the transcription factor *ERF1*. Over-expression of *HDA19* causes *ERF1* induction (Zhou *et al.*, 2005). *ERF1* over-expression is sufficient to induce *PDF1.2* expression and thus leads to resistance to several necrotrophic fungi (Berrocal Lobo *et al.*, 2002; Lorenzo *et al.*, 2003; Solano *et al.*, 1998). *PDF1.2* serves as a useful marker for the activation of ET/JA pathway, but defense responses mediated by ET and JA also involve aspects distinct from *PDF1.2* induction.

The importance of JA against *Phytophthora capsici* Leonian in *Capsicum annuum* was investigated in a study by Ueeda *et al*. (2005). The leaves of this pepper plant were inoculated with *P. capsici* and expression levels of JA and HR related genes were investigated. Results showed that within 0.5 h after inoculation the JA levels had increased more than 14 times when compared to the mock treated plants. Jasmonic acid levels in the susceptible cultivar exceeded those of the control but were not as high as those in the resistant cultivar. The results have shown that JA mediated defense played an important role in the resistance of pepper plants against the hemibiotrophic *P. capsici* (Ueeda *et al.*, 2005).

Ethylene is a gaseous molecule that has been implicated in many physiological and developmental processes which include chlorosis, senescenes, cell death as well as fruit ripening and seed germination (Abeles *et al.*, 1992). Ethylene binds to specific receptors and triggers a phosphor-relay through which two-component signal transducers activate downstream protein kinase cascades, terminating the stimulation of either gene transcriptional activators or repressors (Fluhr, 1998; McGrath & Ecker, 1998). The availability of mutants in ET production of signalling has made it possible to study the role of this hormone in a more functional way (Broekaert *et al.*, 2006). Conflicting results were obtained showing that in some cases ET can act as a virulence factor of bacterial and fungal pathogens and, in other cases, indicating its involvement as a signalling compound in disease resistance (Broekaert *et al.*, 2006; Van Loon *et al.*, 2006a). Interestingly, JA and ET, two very different molecules, with different roles during a plant's life cycle interact in similar

ways and often require concomitant activation during pathogen-specific resistance. Many studies have indicated this synergistic operation to activate the expression of some defense related genes after pathogen inoculation (Glazebrook, 2005; Penninckx *et al.*, 1998; Thomma *et al.*, 2001). Both JA and ET may act in resistance through regulation of ROS, HR, defense genes and the phenylpropanoid pathway, however, much still remains unknown (Knoester *et al.*, 1998).

Several studies have demonstrated that concentrations of JA increase locally in response to pathogen infection or tissue damage and that exogenous application of JA induced the expression of defense related genes (Lorenzo & Solano, 2005; Wasternack, 2007). The importance of ET as a signalling compound was investigated in parsley against *P. parasitica.* Parsley cells that were treated with NPP1 elicitors from *P. parasitica* stimulated ET biosynthesis five-fold within five hours relative to cells that were only mock treated with water (Fellbrich *et al.*, 2002). Over the past decade, several mutants affected in JA signal perception and transduction have been isolated and characterised emphasizing its importance as a signalling molecule in regulating defense responses (Bari & Jones, 2009).

Other Phytohormones

Abscisic acid was initially linked with plant development and abiotic stress, but its involvement in biotic stress has become more evident in the past few years (Asselbergh *et al.*, 2008; Mauch-Mani & Mauch, 2005). It seems that ABA is linked to the signalling network in that it attenuates JA/ET-dependent gene expression and influences JA biosynthesis and resistance caused by JA inducing necrotrophic pathogens (Adie *et al.*, 2007; Anderson *et al.*, 2004; Flors *et al.*, 2008). Abscisic acid governs numerous processes of plant growth and development including seed germination, embryo maturation, leaf senescence, stomatal aperture and adaptation to environmental stresses (Wasilewska *et al.*, 2008). Many studies have indicated the involvement of ABA in plant defense responses (Adie *et al.*, 2007; de Torres-Zabala *et al.*, 2007; Mauch-Mani & Mauch, 2005; Mohr & Cahill, 2007). The exact

role of ABA in defense seems to be intricate due to the different types of plant pathogen interactions occurring. It can, however, be generalized that ABA is more involved in the negative regulation of defense against pathogens (Bari & Jones, 2009).

Exogenous application of ABA increased the susceptibility of plant species such as *Arabidopsis* to *P. syringae pv tomato* (de Torres-Zabala *et al.*, 2007) and soybean to *P. sojae* (Mohr & Cahill, 2001). It is known that ABA plays a role in defense by transcriptional reprogramming of plant cell metabolism (Bari & Jones, 2009). Abscisic acid is known to regulate cell wall metabolism in *Arabidopsis* and recently it was proved that ABA stimulated the expression of a catalase gene (*CAT1*) which is known to be a scavenger of H_2O_2. Abscisic acid was also found to induce H_2O_2 production, which indicated that H_2O_2 might also be involved in ABA induced *CAT1* expression (Bari & Jones, 2009). It seems that *CAT1* might play a role in the feedback regulation of H_2O_2 signalling separately from its ROS scavenging function. Other evidence indicates that ABA regulates defense responses by the effects it exerts on callose deposition, production of ROS and regulation of defense gene expression (Bari & Jones, 2009).

Auxins play a role in almost every phase of plant development and modulates the expression of genes associated with the biosynthesis, catabolism and signalling pathways of other hormones (Paponov *et al.*, 2008). Auxin is involved in the degradation of transcriptional repressors called Auxin/Indole-3-acetic acid (Aux/IAA). These Aux/IAA repressors bind to auxin response factors (ARFs) and suppress the transcription of specific auxin response genes (Leyser, 2006). The role of auxin as a signalling compound is linked through different ways to the SA-JA-ET signalling network. For example, auxin has been shown to influence JA biosynthesis (Nagpal *et al.*, 2005) as well as the expression of genes involved in JA production (Liu & Wang, 2006). Salicylic acid had a repressive effect on auxin responses, inducing efficient defense responses against hemi-biotrophic pathogens such as *Hyaloperonospora arabidopsidis* (Gäum.) Göker, Riethm., Voglmayr, Weiss & Oberw. and *P.*

syringae. This caused a increased resistance against these pathogens (Wang *et al.*, 2007) and for this reason, the antagonistic effect of SA on auxin signalling appears to be an essential component of SA-dependent resistance against hemi-biotrophs. When *Arabidopsis* plants were treated with benzothiadiazole, a SA analog, it resulted in the repression of a number of auxin responsive genes. It was interesting that the majority of auxin inducible genes were also repressed in systemic tissues after the onset of SAR. These results showed that SAR involves the down regulation of auxin responsive genes (Wang *et al.*, 2007). Exogenous application of auxin has been shown to increase susceptibility against pathogens such as *Agrobacterium tumefaciens* (Yamada, 1993), *Pseudomonas savastanoi* (Yamada, 1993) and *P. syringae pv tomato* DC3000 (Halim *et al.*, 2007; Navarro *et al.*, 2006) indicating that that auxin plays a role in the attenuation of defense responses in plants. On the contrary, when auxin responses were blocked, a heightened resistance in plants could be observed indicating that auxin promotes disease susceptibility when applied exogenously whereas repression of auxin signalling could cause an enhanced resistance in plants (Bari & Jones, 2009). Therefore auxin can act as an important component in the signalling network against various biotrophic and necrotrophic pathogens.

Gibberellins (GAs) were also shown to play a role as signalling compounds and influence the SA-JA-ET network. Gibberellins occur abundantly and are produced by many plants, fungi and bacteria (MacMillan, 2001). Gibberellins have initially been neglected with regards to their role in the signalling network of defense. However, recent studies indicated that GA signalling components play a very important role in disease resistance and susceptibility (Bari & Jones, 2009). Gibberellins are mostly known for their involvement in plant growth by controlling the degradation of growth-repressing proteins named DELLA proteins. Navarro *et al*. (2008) suggested that DELLA proteins increase susceptibility to biotrophic pathogens by repressing SA defense responses and promotes resistance to necrotrophic pathogens by regulating the relative strength of the SA and JA signalling pathways (Navarro *et al.*, 2008). Because GA triggers the degradation of DELLA proteins it seems that GA promotes

resistance to biotrophs and increases susceptibility to necrotrophs (Bari & Jones, 2009). Consequently, it was assumed that if the stability of DELLA proteins could be controlled, gibberellins are able to change the SA-JA-ET network and influence the outcome of the immune response. In a study conducted by Achard *et al.* (2008) DELLA proteins were shown to increase the expression of genes coding for ROS detoxification enzymes and in this manner they monitored the levels of ROS after biotic or abiotic stress. To conclude there is a great proportion of emerging evidence that supports the important role of GA and its signalling components in regulating defense responses against various pathogens although the exact mechanism of action remains unknown and needs further investigation.

Brassinosteroids (BRs) are mostly known for their role in cell expansion and division, differentiation and reproductive development. However when BRs were applied to outer leave surfaces of tobacco and rice plants, they were able to provoke a broad-spectrum resistance (Nakashita *et al.*, 2003). Brassinosteroids are recognised by the receptor BRI1, that interacts with BRI1-associated receptor kinase (BAK1) which in turn induces an intracellular signalling cascade that regulates growth and development-related processes (Belkhadir & Chory, 2006). It should be mentioned that the role of BAK1 in the innate immunity appears to be independent of the function of BAK1 in BR signalling (Kemmerling *et al.*, 2007). The link between BR signalling and the SA-JA-ET network has not yet been established. The involvement of BRs in signalling comes from studies where it was shown that BR mediated resistance operates independently from the SA signalling pathway and that it does not require the activation of *PR* genes and production of SA indicating possible cross talk between BR and other hormone pathways. Although, the role of cytokinins (CKs) in plant defense is not understood, there are indications that CKs are involved in the regulation of plant defense responses against some pathogens which still needs to be determined (Bari & Jones, 2009).

Hypersensitive Response

The HR is defined as rapid death of plant cells near the site of infection that ultimately leads to the restriction of pathogen growth (Goodman & Novacky, 1994) and has been proposed to play a crucial role in disease resistance (Heath, 1980). It is known to develop during different plant–pathogen interactions that can vary enormously in physical appearance as well as in the timing at both macro and microscopic scale (Christopher-Kozjan & Heath, 2003; Krzymowska *et al.*, 2007). The number of cells that die during the HR may not be restricted to cells having direct contact with the pathogen. It is so diverse that it occasionally occurs only in a single cell and other times it can spread to large areas throughout the plant so that these necrotic regions are able to completely limit pathogen colonization (Holub *et al.*, 1994).

The HR would favour growth of pathogens with a necrotrophic lifestyle, as their virulence strategy relies on their capacity to kill host cells (Heath, 2000; Yu *et al.*, 1998). Therefore, the HR is believed to be a typical plant defense response active against pathogens with a biotrophic lifestyle although it can also be exploited by necrotrophic pathogens (Glazebrook, 2005; Tyler, 2009). There are several examples of *R-Avr* gene mediated resistance that appear not to involve the HR. These include barley resistance against all races of *Erysiphe graminis* sp. *hordei*, which is conferred by the *mlo* gene (Freialdenhoven *et al.*, 1996) and the potato *Rx* gene mediating resistance to potato virus X (Kohm *et al.*, 1993).

Several cellular changes that occur during a HR include nuclear migration and alteration, cytoskeletal rearrangement and cytoplasmic shrinkage which have been studied in detail (Heath *et al.*, 1997). Later stages of infection are accompanied by membrane dysfunction (loss of ability to be plasmolysed) and progressive vacuolization of the cytoplasm. Membrane disruption has been proposed to be the critical event for cell death (Park, 2005). Key signals in the activation and development of HR appear to be ion fluxes, ROS and SA (Alvarez, 2000; Heath, 2000). The expression of HR is believed to be under strict molecular

control as a form of programmed cell death (PCD), and in many ways resembles animal cell apoptosis (Jones, 2001; Mur *et al.*, 2008).

Elicitins excreted by most *Phytophthora* spp. can elicit a wide range of defense responses in *Nicotiana* spp., including the HR (Kamoun, 2006). Vleeshouwers *et al*. (2008) conducted a study where they expressed a set of 54 effectors of *P. infestans* containing a signal peptide and a RXLR motif that was profiled for the activation of innate immunity on different *Solanum* species. The results showed that the RXLR effector family induced a variety of effector responses of which one was the HR in *S. stoloniferum.*

Phenylpropanoid pathway

The phenylpropanoid pathway is responsible for the production of a wide range of protective compounds against UV resistance, wounding and pathogen attack (Dixon & Paiva, 1995). The rate-limiting enzyme that controls the extent of phenylpropanoid synthesis is phenylalanine ammonia-lyase (PAL) which converts L-phenylalanine to trans-cinnamic acid. After this enzymatic event, a number of specific branch pathways are possible for the formation of a variety of compounds (Dixon *et al.*, 2002). However, all classes of phenylpropanoid compounds are not present in all plant species. Many of the biosynthetic enzymes are encoded by large gene families and the function of many individual family members remains unknown (Dixon *et al.*, 2002).

The regulatory genes and feedback mechanisms that direct the rapid, coordinated induction of phenylpropanoid defenses in response to pathogen recognition, are less well understood (Dixon *et al.*, 2002). Phenylpropanoid biosynthesis pathways are among the most often observed metabolic activities that are transcriptionally induced upon infection with pathogens (Hagemeier *et al.*, 2001). Rapid increase in the expression and enzyme activity of *PAL* and other phenylpropanoid genes is often associated with plant resistance (Habereder *et al.*, 1989). Three phenylpropanoid-derived compounds of particular importance in pathogen-

specific resistance are SA, lignin (cell wall fortification) and some phytoalexins (Dixon & Paiva, 1995).

Isoflavone synthase (IFS) is a key biosynthetic enzyme that forms part of the phenylpropanoid pathway. Silencing of *IFS* in soybean suppressed isoflavonoid phytoalexin accumulation which caused an increase of disease symptoms after soybean was infected with the oomycete *P. sojae* (Subramanian *et al.*, 2005). Suspension cultured parsley cells that were treated with an elicitor of *Phytophthora megasperma* var. *sojae* showed rapid increase in two enzymes that form part of the phenylpropanoid pathway namely PAL and courmarate:CoA ligase (Hahlbrock *et al.*, 1981). These examples clearly prove the involvement of the phenylpropanoid pathway in defense responses.

Pathogenesis related proteins

The term pathogenesis related protein (PR protein) was first used to describe numerous extracellular proteins that accumulated in response to tobacco mosaic virus (TMV) infection of susceptible tobacco genotypes (Hammond-Kosack & Jones, 1996). The definition of a PR protein has since been extended to include intra- and extracellular localised proteins that accumulate in plant tissue after pathogen attack (Bowles, 1990). Pathogenesis related proteins are low-molecular-weight proteins that accumulate at the site of infection and during SAR in distal tissues (Ward *et al.*, 1991). Seventeen main classes of PRs (PR-1 to 17) have been identified based on primary structure, serology, and/or enzymatic or biological activity (Van Loon & Van Strien, 1999; Van Loon *et al.*, 2006b). Several PR proteins possess either antifungal or antibacterial activity *in vitro* (Melchers *et al.*, 1994). In leaves, PRs are present in both epidermal and mesophyll cells, as well as in the vascular bundles whereas with roots they are mostly observed in root epidermal cells.

There are many examples illustrating the involvement of PR proteins in defense response. In a study by Hoegen *et al.* (2002) potato plants were found to accumulate PR-1b in the

epidermal cell layer of the leaf area in response to infection by *P. infestans*. Another important example is the necrosis-inducing *Phytophthora* protein 1 (NPP1) of *P. parasitica* that was infiltrated into the leaves of *Arabidopsis thaliana* Col-0 plants. This resulted in transcript accumulation of *PR* genes. The activation of *PR-1* was found to be SA dependent (Fellbrich *et al.*, 2002). The role of PR proteins in resistance has been supported by transgenic studies of tobacco where high expression of PR genes resulted in resistance to two oomycetes, *Peronospora tabacina* and *P. parasitica* (Alexander *et al.*, 1993).

Among the PR proteins, osmotin and thaumatin-like proteins have been recognised as members of plant PR-5-type proteins (Van Loon & Van Strien, 1999). There are now many lines of evidence indicating that proteins of the PR-5 group from various plant species have *in vitro* antifungal activity against several classes of fungi and oomycetes (Van Loon & Van Strien, 1999). In a study conducted by Foagaga *et al.* (2001), the antifungal properties of tomato *PR-5* were investigated *in vivo* by introducing the tomato *PR-5* coding sequence under the 35S promoter in sweet orange plants. The tolerance of the transgenic plants was tested against *Phytophthora citrophthora* infections. It was proven that these transgenic plants were constitutively expressing the tomato PR-5 proteins and displayed increased protection against this citrus pathogen. This provided evidence of an *in vivo* role of the PR-5 protein in disease resistance (Fagoaga *et al.*, 2001).

PR-2 represents a group of β-1,3-glucanases (Van Loon & Van Strien, 1999), whose role in disease resistance is often related to their glucanase activity. PR-2 can either directly impair the growth of a fungus by hydrolyzing β-1,3/1,6-glucans within fungal cell walls (Mauch *et al.*, 1988) or by releasing short glucan fragments from pathogen cell walls, which can be recognized by plants. PR-9 is classified as a group of peroxidases (Van Loon & Van Strien, 1999). The importance of peroxidase in plant defense was highlighted earlier before the discovery of its role as a PR protein when it was categorized as a PR-9 (Lovrekovick *et al.,* 1968). Peroxidases have been linked with numerous functions. They can contribute to plant

disease resistance in several ways including the strengthening of plant cell walls through the deposition of lignin, which is thought to be a broad spectrum defense mechanism (Vance *et al.*, 1980) and also by cross linking of cell wall proteins (Hiraga *et al.*, 2001). It is also known to play a role as a ROS scavenger (Lamb & Dixon, 1997). High concentrations of ROS can be very toxic to the plant and thus after the oxidative burst has occurred certain genes are activated accordingly to produce enzymes to restore the balance of these oxygen species. Peroxidase is also able to scavenge H_2O_2 by converting it to water and oxygen. García-Pineda *et al.* (2010) investigated the role of peroxidase in avocado after infecting a susceptible rootstock with *P. cinnamomi.* Peroxidases are known to be involved in oxidising phenolic compounds to yield lignin and this is usually confirmed by tissue browning. Although a great increase in peroxidase was observed four days after infection no change in the lignin content was observed and as a result of this it was concluded that peroxidase was not involved in lignin accumulation in avocado.

Phytoalexins

Phytoalexins are low-molecular-weight secondary plant metabolites with antimicrobial properties that accumulate rapidly at the site of attempted pathogen infection (Smith, 1996). The exact role of phytoalexin production in disease resistance has been investigated across a wide range of plant species. Many phytoalexins have been shown to play roles in resistance possibly by serving as disinfectants that help in sequestering infected cells from healthy tissue (Bednarek & Osbourn, 2009). Glyceollin, a phytoalexin of soybean (*Glycine Max* L. Merr) (Ayers *et al.*, 1976), was shown to be the major factor in restricting pathogen growth in a study of the soybean-*P. sojae* interaction where the roles of phytoalexins and other key determinants of resistance were investigated (Mohr & Cahill, 2001). Moy *et al.* (2004) studied gene expression of soybean plants upon infection by *P. sojae* and found that genes encoding enzymes involved in phytoalexin biosynthesis and pathogenesis-related proteins were strongly up-regulated during infection. Substantial evidence has highlighted

the importance of phytoalexins in plant resistance. Capsidiol is one of the major phytoalexins present in pepper plants and is produced by the isoprenoid pathway. This pathway is catalyzed by a sesquiterpene cyclase *(CASC1)* that has synthase activity (Whitehead *et al.*, 1989). Silvar *et al.* (2008) proved a distinct up-regulation of the *CASC1* gene at 24 hpi with *P. capsici,* particularly in the more resistant pepper cultivar that showed an increase of up to six times when compared to the susceptible pepper cultivar (Silvar *et al.*, 2008). Much has been learnt about phytoalexins during recent years. Evidence now exists to show the involvement of these molecules in defense, although further studies are still needed to provide insights into plant pathogen interactions.

CONCLUSION

Avocado is an economical crop worldwide and of great importance especially for South Africa. Various diseases threaten this commercial crop, of which root rot caused by *P. cinnamomi* is the most severe. PRR influences the production and yield of this crop by dramatically reducing the fruit size, causing early fruit drop and eventually leading to tree mortality. Currently, control relies on the use of phosphite injections into tree trunks and often tolerant rootstocks and mulches are used in combination with phosphite in order to achieve optimal results. With cases of insensitivity of *P. cinnamomi* against phosphite being reported, it is only a matter of time before the use of phosphite becomes less effective and the pathogen reaches a phase of complete resistance. Thus the need for other control strategies is currently one of the main focuses of avocado improvement. The use of resistant/tolerant rootstocks is a promising approach. What causes certain rootstocks to be highly tolerant against PRR and other to completely succumb under infection? This question needs to be addressed as there is a lack of understanding regarding defense mechanisms for the interaction between avocado and *P. cinnamomi*.

The avocado - *P. cinnamomi* interaction, although very important not only to science but also to the industry, has received little attention the past few years. With only a few molecular studies undertaken for this interaction, limited information has been gained. Studies by Garcia Pineda *et al.* (2010) highlighted the involvement of a ROS burst as well as the catalaze, epicathecin and NO in the defense response against *P. cinnamomi* within avocado. Muniz *et al.* (2012) used a proteomics approach and were the first to report on protein induction in response to infection of *P. cinnamomi* in roots of avocado. Proteins that were found to be up-regulated included isoflavone reductase, glutathione S-transferase, several abscisic acid-stress-ripening proteins (ASR), cinnamyl alcohol dehydrogenase, cinnamoyl-CoA reductase, cysteine synthase and quinone reductase. Research of García-Pineda *et al.* and Muñiz *et al.* have barely begun to scratch the surface of how defense against *P. cinnamomi* is mediated within avocado especially as this interaction is not thought to have co-evolved. A multigenic trait is more likely as various levels of tolerance in avocado rootstocks against *P. cinnamomi* has been documented.

There is still so much to be discovered if one looks at all the information available on defense responses of model organisms to various pathogens. More molecular studies need to be undertaken as this will also expand knowledge for the basal angiosperm group which is one of the understudied groups in the scientific world that does not constitute a model species. With more information becoming available more research questions regarding this interaction can be answered and more knowledge on the genetic basis of defense responses can be gained. The fast development of new engineering's offers various advantages such as saving time and cost and an exponential increase in data obtained. More information can be gained by studying the histological responses, proteomics, biochemical and genetic pathways as there are various technologies that allow these areas to be studied.

The genetics underlying the tolerant phenotype remains unknown although the search for gene conferring tolerance remains an area of great interest. The identification of defense genes in avocado against *P. cinnamomi* can be valuable as these genes could be used as markers to aid in breeding programmes and could be used for genetic improvement. With markers identified the time required in breeding programmes will be significantly reduced whereby the industry would also benefit.

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FIGURES

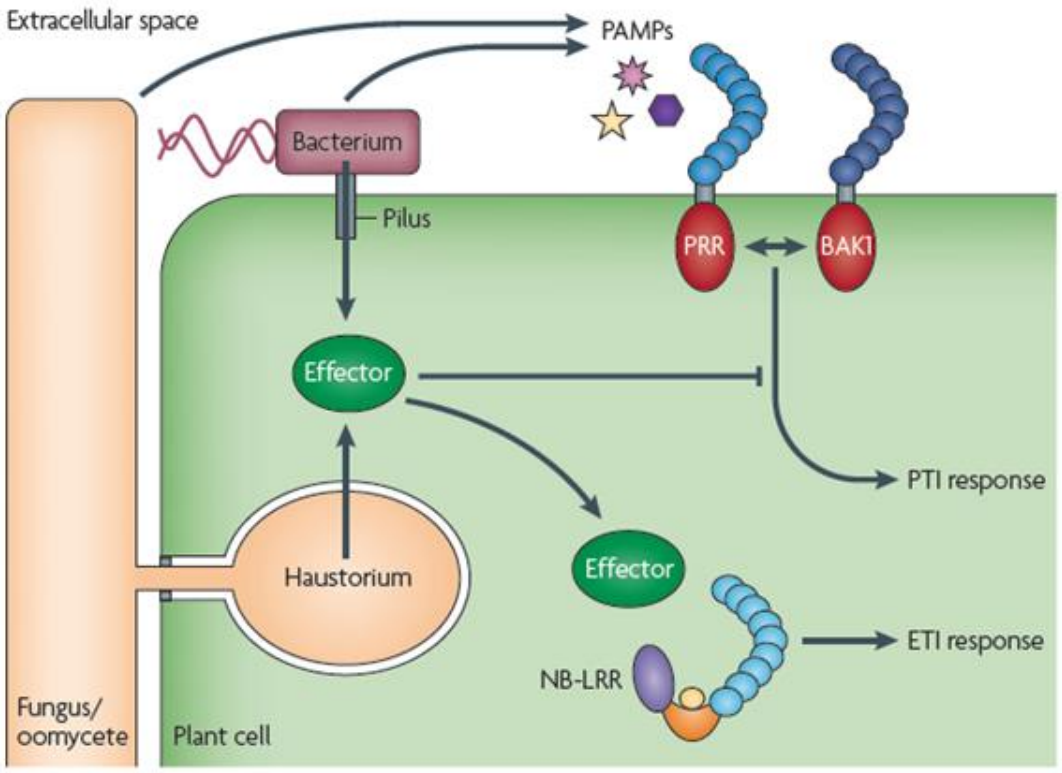

Figure 1. Simplified schematic representation of the plant immune system. Most fungal and oomycete pathogens extend their hyphae into the extracellular spaces of plant issues, although many also form specialized feeding structures, known as haustoria, that penetrate host cell walls but not the plasma membrane. Other fungi extend invasive hyphae into plant cells, but again do not breach the host membrane. Molecules released from the pathogens into the extracellular spaces, such as lipopolysaccharides, flagellin and chitin (PAMPs) are recognized by cell surface pattern recognition receptors (PRRs) and elicit PAMP-triggered immunity (PTI). PRRs generally consist of an extracellular leucine-rich repeat (LRR) domain (mid-blue), and an intracellular kinase domain (red). Fungi and oomycetes deliver effectors from haustoria or other intracellular structures by an unknown mechanism. These intracellular effectors often act to suppress PTI. However, many are recognized by intracellular nucleotide-binding (NB)-LRR receptors, which induces effector-triggered immunity (ETI). NB-LRR proteins consist of a carboxyl-terminal LRR domain (light blue), a central NB domain (orange crescent) that binds ATP or ADP (yellow oval), and an amino-terminal Toll, interleukin-1 receptor, resistance protein (TIR) or coiled-coil (CC) domain (purple oval) (Dodds & Rathjen, 2010).

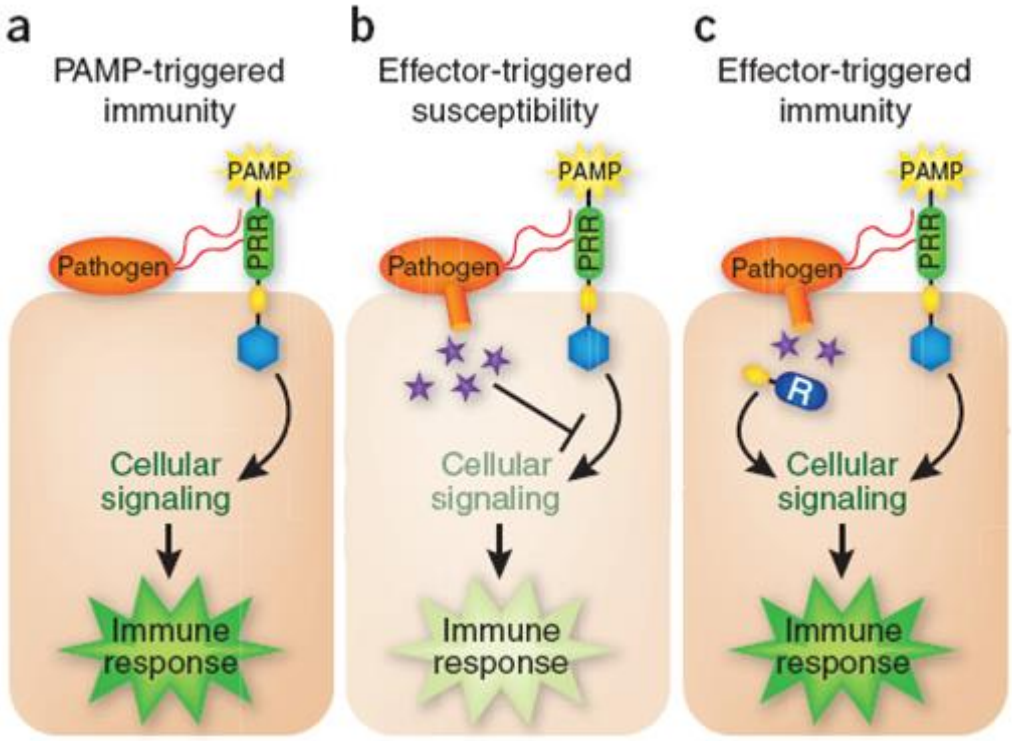

Figure 2. Simplified schematic representation of the plant immune system. (a) Upon pathogen attack, pathogen-associated molecular patterns (PAMPs) activate pattern-recognition receptors (PRRs) in the host, resulting in a downstream signalling cascade that leads to PAMP-triggered immunity (PTI). (b) Virulent pathogens have acquired effectors (purple stars) that suppress PTI, resulting in effector-triggered susceptibility (ETS). (c) In turn, plants have acquired resistance (R) proteins that recognize these attacker specific effectors, resulting in a secondary immune response called effector triggered immunity (ETI) (Pieterse *et al.*, 2009).

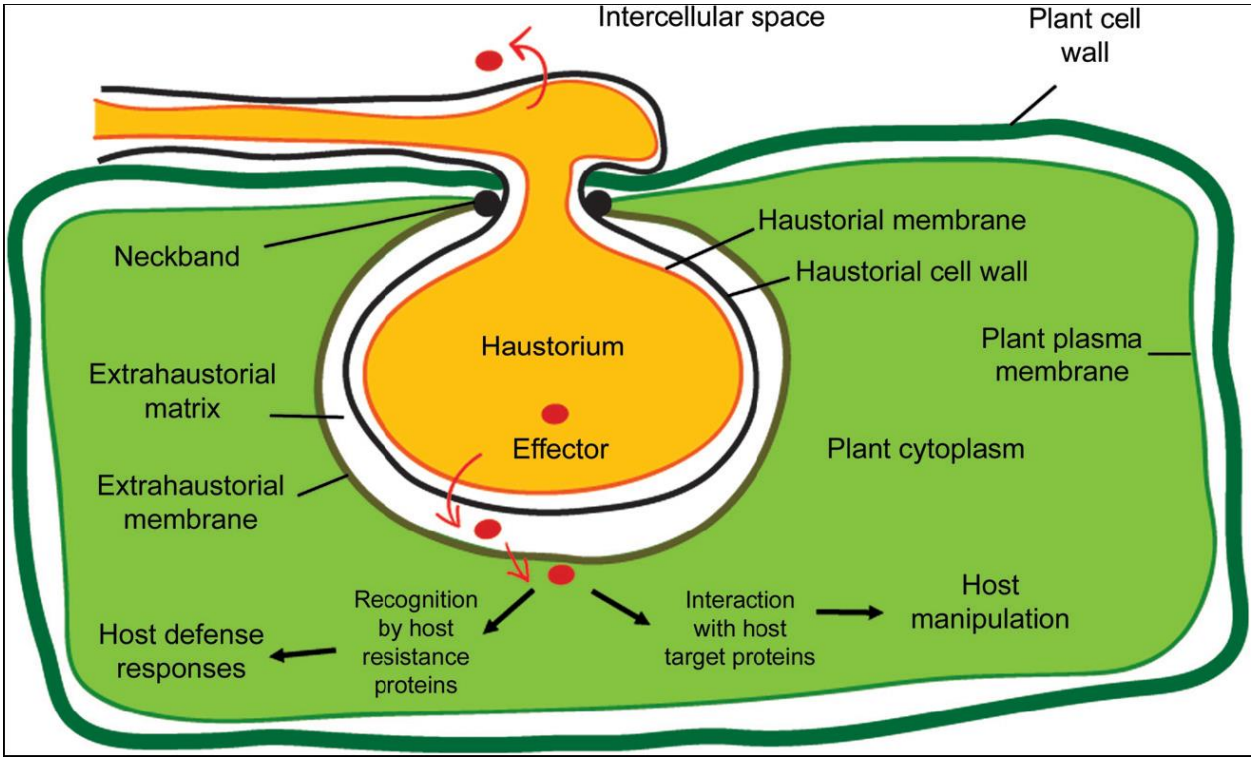

Figure 3. Host–haustorium interactions. During haustorium development, the pathogen penetrates the plant cell wall and invaginates the host plasma membrane. The plant plasma membrane remains intact but becomes specialized in the region surrounding the haustorium; this region is referred to as the extrahaustorial membrane. The region between the haustorial cell wall and the extrahaustorial membrane is the extrahaustorial matrix. Effectors (red dots) are secreted into the apoplast, including the extrahaustorial matrix, and must cross the extrahaustorial membrane (a modified host plasma membrane) before entering the plant cytoplasm, where they may target host proteins to manipulate host metabolism, or can be recognized by host resistance proteins, resulting in the triggering of the host defense response.

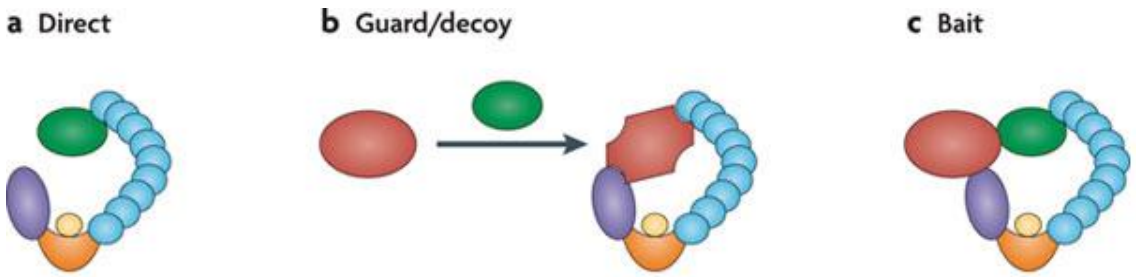

Figure 4. Direct and indirect effector recognitioning. Plant nucleotide-binding (NB)-leucine-rich repeat (LRR) receptors can recognize pathogen effectors by either direct or indirect mechanisms. a) In direct recognition, the effector (green) triggers immune signalling by physically binding to the receptor (purple, orange, yellow and blue) b) In the guard and decoy models, the effector modifies an accessory protein (red), which may be its virulence target (guard model) or a structural mimic of such a target (decoy model). The modified accessory protein is recognized by the NB-LRR receptor. c) Under the bait model, interaction of an effector with an accessory protein facilitates direct recognition by the NB-LRR receptor (Dodds & Rathjen, 2010).

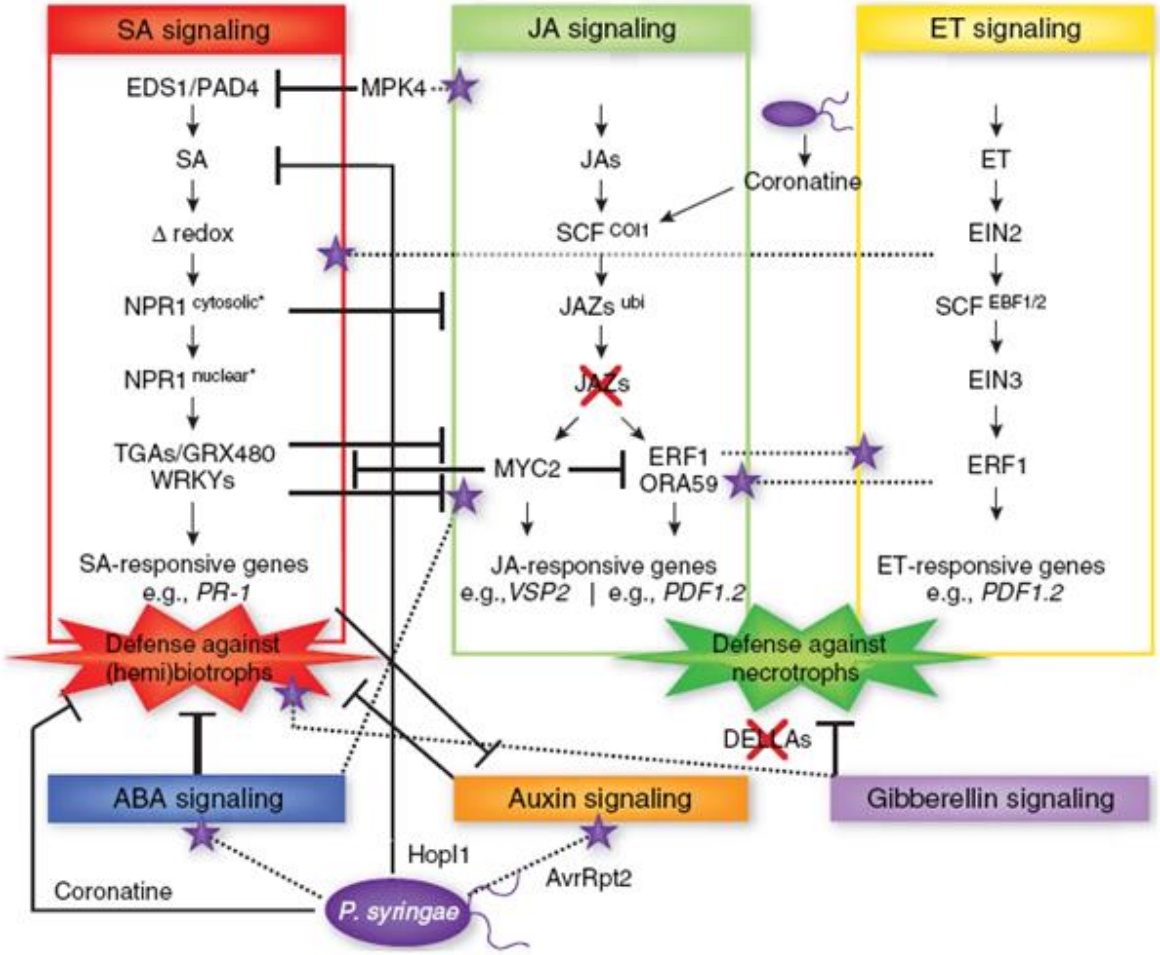

Figure 5. Networking by phytohormones in the plant immune response. Cross-communication between hormone signalling pathways provides the plant with a large regulatory capacity that may tailor its defense response to different types of attacker. On the other hand, pathogens such as *P.syringae* produce effector proteins that manipulate the signalling network to suppress host immune responses and promote virulence. The SA, JA and ET signalling pathways represent the backbone of the defense signalling network, with other hormonal signalling pathways feeding into it (Pieterse *et al.*, 2009).

CHAPTER 2

Development of two small-plant assays for studying the avocado – *Phytophthora cinnamomi* interaction

ABSTRACT

The oomycete *Phytophthora cinnamomi* is known to cause root rot on more than 3000 plant species worldwide, of which avocado is an agricultural important crop that is severely affected. Due to the lack of resistant avocado rootstock varieties, large economic losses occur frequently. The pathogenesis of *P. cinnamomi* and the molecular basis of plant responses against *P. cinnamomi* are poorly understood. The objective of this study was to establish novel pathosystems to help with our understanding as well as the assessment of the interaction between *P. cinnamomi* and the plant *Persea americana*. In this study two pathogenicity screening systems were developed, namely a perlite solid growth substrate that could be used for long term disease assessment and a hydroponic system to provide root material for downstream applications. Inoculation of avocado roots with *P. cinnamomi* from both systems consistently produced visible disease symptoms typical to that of Phytophthora root rot. *Phytophthora cinnamomi* was re-isolated from infected roots from both systems, yielding consistent infection. The ease of access to the root system in the hydroponic system allowed roots to be sampled with limited damage and proved to be efficient for downstream studies such as microscopy and molecular applications. The solid substrate system proved to be invaluable to evaluate disease resistance in plants over a six week period and could also be used for *in planta* quantification and detection studies of the pathogen. These two pathosystems were valuable to study and unravel the molecular basis of plant responses between avocado rootstocks and *P*. *cinnamomi.*

INTRODUCTION

Despite many advances in plant pathology basic plant pathogenicity assays remain important when assessing disease resistance in plants against phytopathogens. The availability of reliable methods for screening and evaluation for improving tolerance against pathogens are an invaluable tool in breeding programmes. In addition to screening for disease resistance, the plant material is an important resource for downstream applications such as microscopy and molecular biology.

Many factors influence the development of a reliable assay such as plant uniformity, variation, number of replicates, inoculation source and concentration, rating of disease incidence and severity as well as environmental greenhouse conditions. Greenhouses offer the advantage of a regulated environment that provides favourable conditions for disease development and evaluation whereas the onset of disease under field conditions can be suppressed due to environmental conditions which can lead to under-estimation of the disease severity caused by a pathogen.

Phytophthora cinnamomi Rands is the causal agent of Phytophthora root rot (PRR) on a number of important agricultural and forestry crops including avocado (Hardham, 2005). This oomycete continues to threaten crop yields as well as native vegetation such as the undisturbed fynbos vegetation of the unique floral kingdom of the Cape province in South Africa (von Broembsen & Kruger, 1985). In avocado production PRR is regarded the most limiting biotic factor influencing plant health. *Phytophthora cinnamomi* has the potential to destroy avocado orchards and therefore rapid identification and subsequent disease management is essential. One of the most convenient, inexpensive and environmentally sound ways to control plant disease is to utilize disease resistant varieties (Agrios, 2005). Avocado is one such crop where the development/selection of rootstocks with resistance/tolerance to PRR is vital for successful production globally. Currently the industry relies on the use of phosphite trunk injections and highly tolerant rootstocks to combat PRR.

Avocado rootstocks play a very important role not only in tree vigor and production but also in resistance to certain fruit diseases (Wolstenholme, 2003).

Currently a mist bed screening method is used by Westfalia Technological Services (WTS) in their selection program that screens for PRR tolerant avocado rootstocks. This selection technique involves the use of vermiculite as growth substrate so that infection is carried out by using mycelia of a virulent strain of *P. cinnamomi* since the production of zoospores is rather difficult. Mycelia are mixed with vermiculite, after which avocado plantlets are transplanted into a mist bed without any wounding. After six weeks avocado seedlings are evaluated. Seedlings that displayed more than 60% healthy roots are put through a second screening test. Only seedlings with better root health than 'Duke 7' or 'Dusa®' are selected from the second mist bed screening, vegetatively propagated and grafted with 'Hass' and transplanted into a heavily infested field where PRR tolerance is evaluated over several years (Kremer-Köhne *et al.*, 2011). This method is time consuming, labour intensive and disease development tends to take much longer. Currently, it takes approximately 25 years for a specific avocado rootstock to be commercialised through breeding programs (Kremer-Köhne *et al.*, 2011).

Currently, no published literature is available describing a reliable greenhouse assay for studying the interaction between avocado and *P. cinnamomi.* There is a great need for such a system especially to assess root symptoms upon infection as well as for obtaining clean root material for microscopy and downstream molecular processes such as sequencing and quantitative gene expression analysis. The objective of this study was therefore to establish two small plant assays that could be used to address these basic needs. The first was a system that can be used for long term resistance screening and for assessment of root rot symptoms. A second system was evaluated for its efficiency to provide easy access to clean roots without any contaminants adhering to it. For the first pathosystem, two solid substrates namely vermiculite and perlite were evaluated and for the second system a hydroponics

system was modified. Three rootstock varieties known to be highly tolerant, tolerant and less tolerant to *P. cinnamomi* respectively were evaluated to verify the efficacy of the inoculation technique.

MATERIALS AND METHODS

Koch's postulates

To develop a reliable pathogenicity assay it is essential that the four postulates of Koch are met. The aim of Koch's postulates is to confirm that a particular organism is the causal agent of a specific disease. Firstly, the pathogen should be associated with the host; secondly it should be isolated from diseased plants and grown in pure culture. Thirdly the isolated pathogen should then be used to inoculate healthy plants of the same species from where it was isolated and lastly the pathogen should be re-isolated from the new diseased plant to prove Koch's postulate (Evans, 1976).

Plant material

Three avocado rootstocks were selected based on the status of their tolerance to *P. cinnamomi*. These rootstocks included R0.09 (highly tolerant), R0.10 (tolerant - industry standard 'Duke 7') and R0.12 (less tolerant) and were produced by WTS (Tzaneen, Limpopo province, South Africa). The clonally propagated plantlets were nine months old and were transplanted into vermiculite and perlite respectively, in 1.5 L plastic bags. For the solid growth substrate system, approximately six weeks after the plants were transplanted and recovered from stress; 30 plants were removed from bags to be inoculated and replanted respectively in either vermiculite or perlite. A hydroponics system previously used for banana (Van Den Berg *et al.*, 2007) was adapted for the avocado - *P. cinnamomi* interaction. For the hydroponics system, 27 plants were removed from their bags and transplanted into 500 ml polystyrene cups filled with water to ensure easy access to the roots.

Inoculum preparation

A *P. cinnamomi* isolate provided by WTS was used as the inoculum source. This isolate was initially isolated from diseased avocado feeder roots that were collected from an infected avocado orchard. Feeder roots were dipped in 70 % ethanol followed by H_2O and small pieces containing diseased and healthy root tissue were cut and placed in selective NARPH-V8 media (20 g agar, 200 ml filtered V8 broth, 800 ml deionized water, 50 g hymexazol, 10 mg rifampicin, 250 mg ampicillin, and 125 mg a.i. pentachloronitrobenzene) for five days at 25 ºC. DNA was extracted using the method developed by Brunner *et al.* (2001) and the identity was confirmed by amplifying the species specific LPV3 fragment: LPV3-for (5' GTGCAGACTGTCGATGTG 3') and LPV3-rev (5' GAACCACAACAGGCACGT 3') (Kong *et al.*, 2003). Briefly, the 20 µl PCR reaction contained 2.5 µl 10X PCR reaction buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 0.25 µM of each specific primer, 1 U FastStart *Taq* polymerase (Bioline Ltd., London), and 20 to 50 ng of template DNA. PCR cycling conditions were: an initial denaturation step at 94 ºC for 2 min, followed by 30 cycles of 94 ºC for 30 sec, 58.5 ºC for 30 sec and 72 ºC for 1 min and a final extension step at 72 ºC for 7 min. PCR products were separated and examined by electrophoresis on a 2% agarose gel stained with GelRed (Biotium, Inc., California, USA) and visualized under UV light.

Zoospore suspension

Phytophthora cinnamomi was first grown on V8 agar plates (50 ml of filtrated V8 juice, 0.5 g CaCO3, 15 g agar, 1 liter distilled water, autoclaved at 121°C for 15 minutes) for five days. Small agar blocks (10 mm x 5 mm) containing mycelia were cut from the actively growing margin of the plates and transferred onto empty 90-cm-diameter Petri dishes, to which 25 ml of 2% V8 broth (20 ml V8 juice with 0.2 g $CaCO_3$ in 1 L dH_2O) was added and left for at least 3 days at room temperature (ca. 25 ºC). The broth was removed and agar blocks containing mycelia were rinsed three times with H_2O after which 25 ml of filtered stream water was added and incubated for 2 – 3 days at room temperature under UV light. Sporangia

formation was monitored during this incubation step and once enough sporangia were observed, the plates were cold shocked by placing them at 4 ºC for 45 min after which they were removed immediately and left at room temperature for 1 hour to stimulate zoospore release. The zoospore suspension was removed from the plates, pooled and used for inoculation for both systems.

Inoculation

Solid growth substrate (Perlite and Vermiculite) system

Roots were suspended for an hour in a 5 L cake saver containing zoospores at a concentration of 5.8 x 10^4 ml^{-1} after which they were transplanted into 1.5 L plastic bags filled with perlite and vermiculite, respectively. Of the remaining suspension, 25 ml was added to each plant. Only sterile water was added to each of the control plants. Six weeks after inoculation, plants were evaluated for root rot symptoms.

Hydroponics system

The avocado plantlets were inoculated with both mycelia and zoospores. Plant roots were suspended in 500 ml polystyrene cups. Zoospores at a concentration of 1.5 x 10^3 ml^{-1} and 50 ml mycelial suspension were added to each polystyrene cup. Mycelia suspension was added due to the low concentration of zoospores that was obtained. Only sterile water was added to cups containing the control plants. A subset of infected plants were removed from water at 96 hours post infection and transplanted to 1.5 L plastic bags filled with perlite. After six weeks these plants were evaluated to confirm successful infection.

Evaluation of PRR disease severity in three avocado rootstocks grown in solid substrate.

Six weeks after infection, rootstocks were evaluated for their tolerance to *P. cinnamomi.* Disease assessment was only carried out for the solid growth substrate system using perlite

as this system was selected to be used for long term resistance screening. Three rootstock varieties were planted in a completely randomized block design. Ten plants of each variety were included. The development of symptoms on avocado plants was rated using a combination of two disease severity rating scales used by Gabor *et al*. (1990) and Ivors *et al*. (2008). According to this scale plants are scored based on their foliar symptoms as well as their root symptoms using a rating scale ranging from 0 to 5, respectively. Plants with no foliar symptoms were given a value of 0 and plants completely necrotic a value of 5. For root symptoms, plants with healthy roots scored 0 and plants whose roots were completely necrotic scored 5 (Table 1). Percentage of disease severity was calculated for each variety using the formula of (Sherwood & Hagedorn, 1958), whereby:

Disease severity (%) = [∑ (No. plants in a disease severity category)(Specific disease scale category)/(Total no. of plants in the trial)*(Maximum disease scale category)]*100*

Data from the disease severity assessment was analyzed using ANOVA and the software package of JMP 9 using a Student's t-test. In all cases significance was evaluated at $P < 0.05$.

RESULTS

Koch's Postulates

Phytophthora cinnamomi was isolated from diseased avocado roots and the identity of the isolate was confirmed using LPV3 specific primers that amplified the expected product of 450 bp (Fig. 1). Pure cultures displayed the characteristically coralloid-type mycelium with prolific hyphal swellings of *P. cinnamomi* (Fig. 2). Healthy avocado plants were infected and six weeks after infection *P. cinnamomi* was re-isolated from diseased roots of these once healthy plants providing sufficient evidence for Koch's postulates (Fig. 1 & Fig. 2). Avocado plants infected with *P. cinnamomi* developed typical root rot symptoms. These symptoms included necrotic lesions that developed on the roots and lower stem, some of the feeder

roots were black and brittle. The newly developed symptoms of the artificially inoculated avocado seedlings correlated with symptoms observed on feeder roots from trees in diseased avocado orchards.

Solid growth substrates (vermiculite and perlite)

Symptoms, typical to that of PRR, developed on the roots of avocado plants, six weeks after infection. The symptoms were very similar when using perlite or vermiculite as growth substrate. These symptoms included necrotic lesions that developed on the leave surfaces (Fig. 3.A), leave discolouration and stunting of leave tips (tip necrosis) (Fig. 3.C) and typical leaf drop (Fig. 3.D). The tolerant rootstock R0.09 did not show any aboveground symptoms when compared to less tolerant R0.12 and fairly tolerant R0.10 rootstocks (Fig. 3.B & Fig. 3.E). At six weeks the infected avocado seedlings showed discoloration and necrosis of the roots. Feeder roots of avocado plants in all systems displayed infection visible as black and brittle root tissue (Fig. 4.C-E). The roots of tolerant R0.09 plants in both perlite and vermiculite were less affected by the pathogen when compared to the more susceptible rootstock (Fig. 4.E). *Phytophthora cinnamomi* was able to infect and cause disease in avocado roots grown in perlite and vermiculite growth substrates. The infection was consistent and disease symptoms that developed on rootstocks with varying levels of PRR-tolerance were consistent with the known levels of tolerance. Root quality was suitable for downstream application with the only constraint of particles being present on the roots (Fig. 4.A & Fig.4.B) which interfered with RNA extraction although good RNA samples were obtained when perlite particles were washed off with water prior to harvesting.

Hydroponics system

The onset of visual disease symptoms on avocado roots grown in the hydroponics system was delayed. By 96 hours post infection plants showed mild infection when compared to plants grown in perlite and vermiculite. At 96 hpi a few infected and uninfected plants were

removed from the hydroponic assay and transplanted into plastic bags filled with perlite. Six weeks after infection these plants showed symptoms similar to those described in the above section for the solid growth substrates. *Phytophthora cinnamomi* was subsequently isolated from infected roots indicating that infection in a hydroponics system was successful.

The hydroponics system allowed roots to be accessed easily and harvested immediately with no particles adhering to the roots. Trial RNA extractions were performed and good quality RNA samples were obtained that could be used for downstream molecular experiments (Fig. 5). Control plants that remained in the hydroponic system (six weeks) displayed typical water logging symptoms where roots started to decompose. This indicates that plants are negatively affected when suspended too long in water.

Confirmation of PRR-susceptibility in three avocado rootstocks

Disease assessment carried out for the perlite growth substrate system indicated that R0.09 plants were the least affected by *P. cinnamomi,* displaying no foliar symptoms when compared to roots that showed moderate infection. From the results it is evident that R0.09 was the most tolerant as indicated by an overall disease severity of 35 % whereas R0.12 was the most susceptible with a disease severity of 93 % (Fig. 6.B). R0.10 had a moderate disease severity of 64 % (Fig. 6.B). Root disease severity was significantly greater in comparison to foliar disease severity (Fig. 6.A).

DISCUSSION

In this study we developed a reliable and repeatable small plant inoculation assay to study PRR of avocado under greenhouse conditions. This in turn allowed for the use of high quality root tissue for downstream applications such as histology and molecular biology. Since the varying levels of tolerance of various avocado rootstocks is of interest, great attention is therefore given to the establishment and use of reliable small plant assays. A reliable pathosystem is of crucial importance for any host-pathogen interaction. Several

researchers in our group have already utilized these assays to study various aspects of the *P. cinnamomi* / avocado interaction. Examples include histopathological evidence of the host's response to infection (Christie, 2012), biochemical assays, the identification and expression profiling of defense-related avocado transcripts as well as the quantification of pathogen DNA *in planta* (see chapter 4).

The two small plant systems included plants infected and grown in vermiculite or perlite (solid substrates) and plants infected and submerged in small cups containing water. Avocado plants grown in the solid substrate allowed for the assessment of disease development / severity over a six-week period. Root material was suitable for visual disease assessment. It is known that vermiculite is an effective substrate for screening of avocado plantlets for PRR-resistance (Kremer-Köhne (2011). In this study we attempted to find an alternative substrate to vermiculite, since vermiculite pieces were found to interfere with RNA extraction from roots. Perlite as a growth substrate proved to be successful for long term resistance screening, and was suitable for studying RNA, DNA, proteins and histology. Excess perlite could be easily removed by rinsing with water, and allowed for successful extraction of DNA, RNA and proteins.

A solid growth substrate has the advantage that it provides the opportunity for plants to readily take up nutrients (depending on the specific growth substrate used), water, oxygen as well as mechanical support to the below-ground plant parts. Growth substrates could easily become contaminated and may contain insects, nematodes or unwanted microorganisms and therefore sterilization might be necessary before use. Nutrient or pH imbalances take much longer to correct and plants grow slower in soil than in hydroponics (Lamberts, 1981).

In this study, vermiculite retained water very well and this could be a disadvantage for mimicking flooding conditions. Artificial substrates are most commonly used in greenhouse crop production (Nelson, 1997). These substrates are made of various components blended

in varying proportions to produce a substrate with physical and chemical properties suitable for its intended use (Bunt, 1988). Perlite is very popular as it decreases soil density when mixed with soil. Perlite and vermiculite are very similar but perlite tends to hold more air and less water whereas vermiculite is known to retain more water (Jackson, 1974). Perlite is composed of a fusion of granite, obsidian, pumice and basalt providing some nutrition whereas vermiculite does not contain carbohydrates, lipids or proteins and consequently the addition of a nutrient solution was necessary (Jackson, 1974).

Avocado plants grown in the hydroponics system provided easy access to the roots without causing excess wounding or damage and disturbing pathogen propagules on root surfaces. This is especially valuable for microscopy and gene expression studies. When conducting downstream applications such as qRT-PCR, obtaining good quality RNA is imperative and therefore a reliable system that provides easy access to avocado roots with minimum contaminants adhering to the roots is needed. Such a system should ensure that roots can be harvested quickly and processed easily without influencing the quality of RNA. Avocado seedlings that are grown in a composted bark soil substrate or vermiculite are less desirable as severe damage is caused to roots in an attempt to gain access. This would give unreliable results in experiments such as qRT-PCR were wounding / damage to roots can induce the expression of genes not related to the specific treatment under investigation and could lead to false results and conclusions. Test RNA extractions confirmed that good quality and quantity RNA could be obtained. The hydroponics system would be the preferred plant assay for strategies that would entail gene expression (see Chapter 3) or sequencing of transcriptomes.

In the hydroponics approach, the onset of disease symptoms was not as visible when compared to vermiculite and perlite. This could be ascribed to the fact that a much lower concentration of zoospores was used in the hydroponics system. We suggest that when using hydroponics, avocado plants should not be suspended in water too long as it causes

hypoxic conditions and plants suffer severely. This was evident from control plants that started to decompose at six weeks whereas a subset of control plants that were transplanted to perlite remained healthy. Hydroponic systems are becoming very popular as it presents various advantages over solid growth substrate systems such as: no soil is needed; water stays in the system; it tends to give high and stable yields; pests and diseases are easier to control when compared to soil systems (Gibeaut *et al.*, 1997) but most importantly and especially with reference to this study is the instant access that is offered to the root system. This is especially valuable for infection studies and monitoring of plant – pathogen interaction as well as easiness of harvesting. The major disadvantage of a hydroponics system is that without soil as a buffer, any failure would lead to rapid plant death (Lamberts, 1981).

Both the hydroponic and perlite inoculation systems were successful in *P. cinnamomi* infection and establishment. The pathogen could be re-isolated six weeks after infection from roots of both systems. The different growth media did not affect the ability of *P. cinnamomi* to infect avocado roots. Both assays proved to be valuable for investigating the interaction between avocado and *P. cinnamomi.*

The pathogenicity trial clearly demonstrated that R0.09 is the most tolerant rootstock against *P. cinnamomi*, but also that *P. cinnamomi* is pathogenic on the avocado host as proven by the disease severity of 93 % in the susceptible R0.12 rootstock. Roots were affected more severely than foliar parts which are characteristic of this pathogen. However with both disease severity rating scales, R0.09 proved to be the most tolerant followed by R0.10 known to be the industry standard. Our results are in agreement with findings of WTS who found R0.09 to be highly tolerant, R0.10 tolerant and R0.12 the least tolerant to *P. cinnamomi*.

CONCLUSION

Establishing a pathosystem is a fundamental building block for gaining insight into the complex interaction occurring between a plant and a pathogen. With the establishment of these two reliable inoculation assays we are now able to study the interaction between avocado and *P. cinnamomi* in the laboratory as successful infection was obtained in all systems evaluated that resulted in typical PRR symptoms. Evaluation of rootstock varieties confirmed the known tolerance status of these rootstocks against *P. cinnamomi* which only provides us with further confidence in our system as it is able to mimic natural infection occurring in the field as well as the well-proven artificial infection used by WTS.

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FIGURES AND TABLES

Table 1. Disease rating scales used to evaluate above- and below- ground disease severity of *Phytophthora cinnamomi* in avocado varieties.

Disease severity	**Foliar disease symptoms on avocado**
0	No disease symptoms visible, healthy
1	Slight chlorosis
2	Moderate chlorosis/necrosis
3	Severe chlorosis/necrosis
4	Complete chlorosis/necrosis
5	Dead plant
Disease severity	**Root disease symptoms on avocado**
0	No disease symptoms visible, healthy
1	20% of root system necrotic
2	40% of root system necrotic
3	60% of root system necrotic
4	80% of root system necrotic
5	100% of root system necrotic

(Gabor *et al.*, 1990; Ivors *et al.*, 2008)

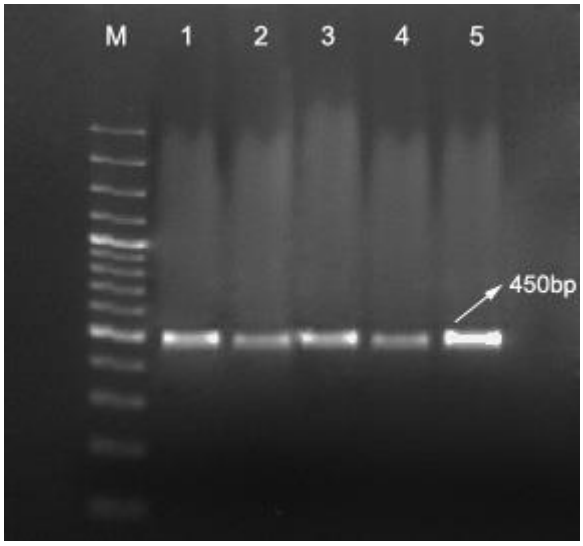

Figure 1. Agarose gel electrophoresis of PCR products for identification of *Phytophthora cinnamomi.* using LPV3 species specific primers. M: 100-bp molecular weight standard; Lane 1 – 3: 450 bp product from DNA that was isolated from cultures obtained from WTS, Lane 4 – 5: 450 bp product from DNA that was isolated from infected avocado roots.

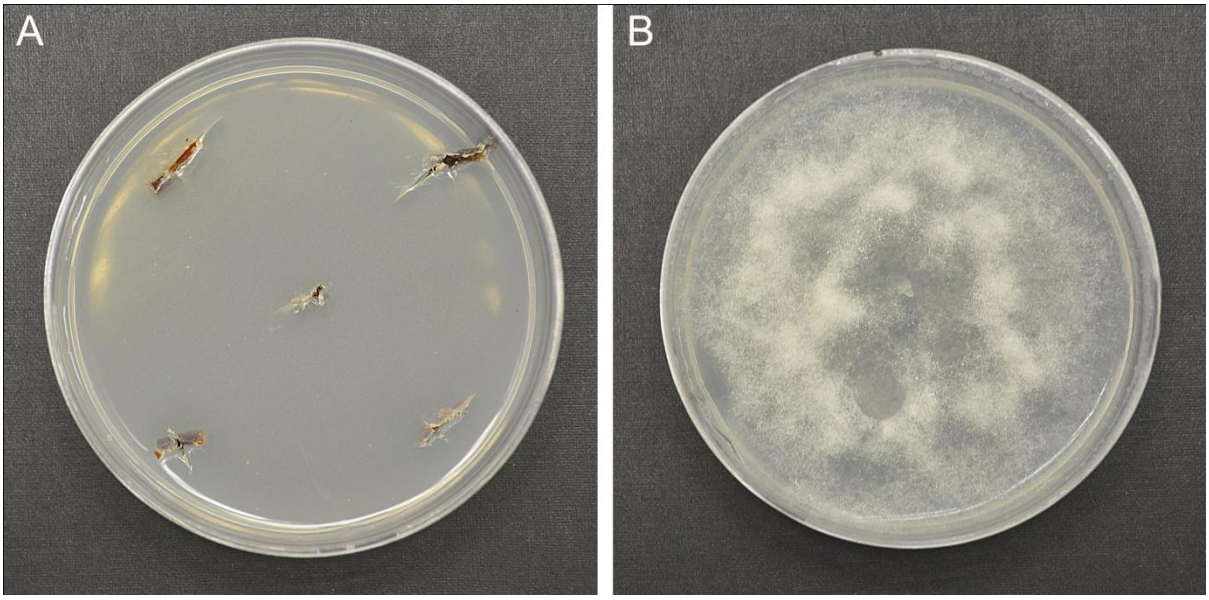

Figure 2. A. Infected root pieces from avocado plants placed in selective NARPH-V8 media after infecting with *Phytophthora cinnamomi.* B. Typical coralloid-type mycelial growth of a pure culture of *P. cinnamomi* grown on ½ PDA that was isolated from infected root pieces.

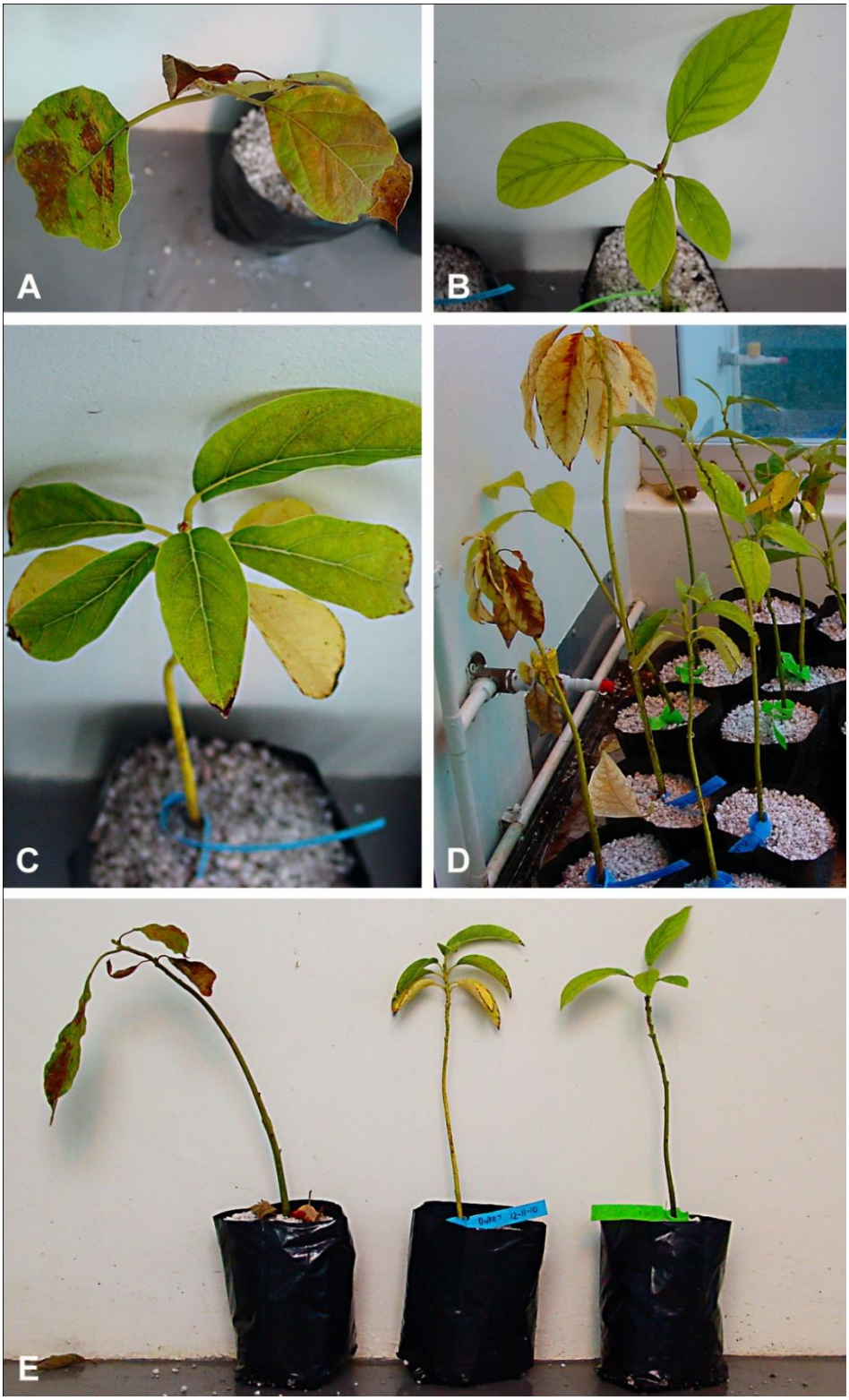

Figure 3. Symptom development of *Phytophthora cinnamomi* infection on avocado rootstocks: A. Necrotic lesions of leaf surfaces B. R0.09 displaying no foilar symptoms C. Tip necrosis D. Leaf drop E. Foliar symptoms of each roostock from left R0.12, R0.10 and R0.09 from the perlite solid growth substrate system.

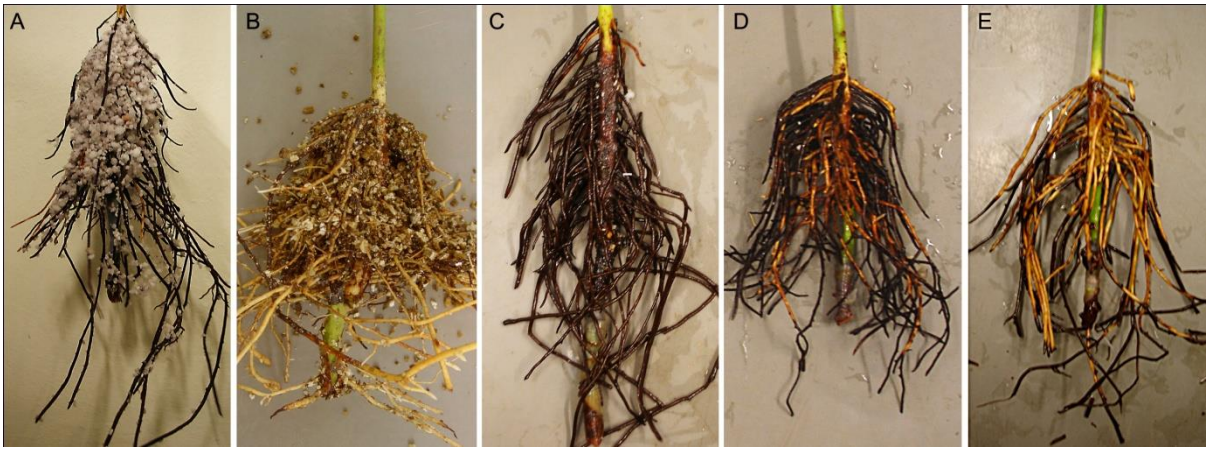

Figure 4. Root symptoms of *Phytophthora cinnamomi* infection on avocado rootstocks using vermuculite and perlite as growth substrates: A. Infected roots grown in perlite B. Roots from control plants grown in vermuculite. C. R0.12 displaying 100% necrotic roots D. R0.10 displaying 80% necrotic roots E. R0.09 with 40% necrotic roots.

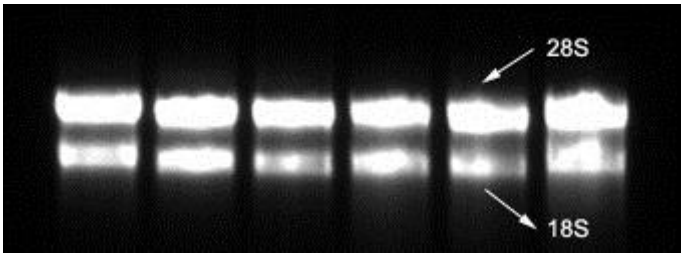

Figure 5. Total RNA from avocado roots as revealed by non-denaturing gel electrophoresis that shows the 28 and 18S ribosomal bands. RNA was extracted from avocado plants using the hydroponic system.

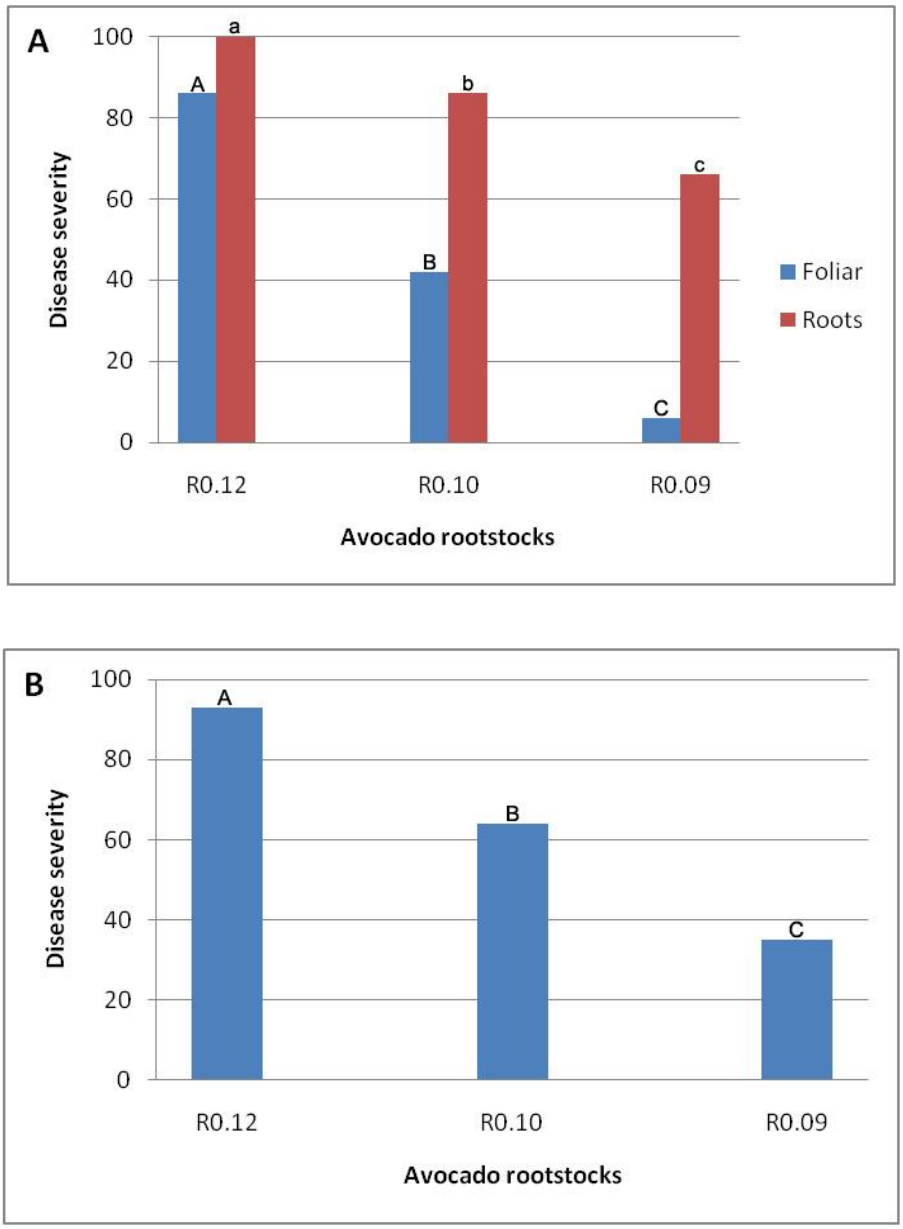

Figure 6. Evaluation of Phytopthora root rot severity in three different avocado rootstocks: R0.09 (highly tolerant), R0.10 (tolerant) and R0.12 (least tolerant). A. Percentage disease severity of *Phytophthora cinnamomi* infection on foliar and root tissues respectively. B. Percentage disease severity when foliar and root ratings were combined. Data were combined so that plants scored a mark out of 10 rather than just 5 for roots and foliar symptoms respectively. Data were analyzed using ANOVA and the software package of JMP 9 using a students t-test. Bars represented with the same letter are not significantly different at $P>0.05$.

CHAPTER 3

Expression of defense related genes against *Phytophthora cinnamomi* in five avocado roootstocks *(Persea americana)*

ABSTRACT

Avocado (*Persea americana*) is one of the major fruit crops worldwide. Various diseases threaten this commercial crop, of which root rot caused by *Phytophthora cinnamomi* is the most severe. The pathogen is known to infect the plant via the feeder roots leading to branch dieback, and eventually tree mortality. While it is known that different avocado rootstocks have varying degrees of susceptibility to Phytophthora root rot (PRR), little research has been done on the avocado-*Phytophthora* interaction. In this study, expression levels of defense-related genes coding for phenylalanine ammonia-lyase, lipoxygenase, pathogenesis-related protein 5, endochitinase, gluthathionine S-transferase, heat shock protein and metallothionein were characterized and compared among five rootstocks with varying susceptibility to PRR, after infecting with *P. cinnamomi*. Root samples were collected at 0, 3, 6, 12, 24, 48 and 72 hours post-infection and expression levels of these genes were determined using quantitative real-time RT-PCR. The results indicated the involvement of *PR-5 and endochitinase* in the defense response of all avocado rootstocks to *P. cinnamomi* but could not be directly linked to the observed phenotypic tolerance. *PR-5* and *endochitinase* were highly up-regulated at 72 hpi. Differences in expression of *Phenylalanine ammonia-lyase* and *lipoxygenase* genes were seen when comparing tolerant and less tolerant rootstocks which may suggest that expression of these genes contribute to tolerance. These data have provided important insights to plant defense and to how different avocado rootstocks may show increased tolerance to infection by *P. cinnamomi*.

INTRODUCTION

Avocados (*Persea Americana* Mill) are susceptible to a wide range of pathogens and pests and therefore it is not surprising that Phytophthora root rot (PRR) is one of the most damaging diseases severely affecting the crop worldwide. The global destructiveness caused by *Phytophthora cinnamomi* Rands has made it one of the most economically important groups of plant pathogens in the world and also one of the most studied. *Phytophthora cinnamomi* is a soil borne pathogen that was first described by Rands in 1922 when the pathogen was isolated from cinnamon trees in Sumatra (Zentmyer, 1983). Primary infection by *P. cinnamomi* occurs at the small absorbing avocado feeder roots resulting in a brownish black and brittle appearance. There is almost no progression into the larger roots (Zentmyer, 1980). This root rot leads to death of the feeder roots which results in insufficient water and nutrient uptake, which eventually leads to tree mortality (Pegg, 1991).

Although PRR has been studied for more than 60 years, total control remains elusive and economical losses continue to increase. Currently control is achieved by spraying or injecting trees with phosphite in conjunction with using tolerant rootstocks. However in 2008, Dobrowolski *et al.* showed decreased pathogen sensitivity to phosphite in Australian avocado orchards where phosphite had been used against *P. cinnamomi* (Dobrowolski *et al.*, 2008). Although this decrease is currently not viewed as a major threat, the potential impact of such a phenomenon on the effectiveness of phosphite for PRR control may be severely negative. In addition to chemical control much emphasis is currently placed on the selection and use of tolerant avocado rootstocks.

Tolerant rootstocks offer the greatest possibility of a sustainable long-term solution for root rot. Several breeding and selection programs around the world have identified rootstocks with a high degree of tolerance to *P. cinnamomi* (Menge & Marais, 2000). 'Duke 7' was discovered by Zentmyer and in 1975 it became the first commercial rootstock with moderate tolerance against the pathogen. It was highly successful and is still used worldwide, although

several newer varieties have been selected since then (Zentmyer, 1980). The fact that 'Duke 7' can acclimatize to many different environmental conditions makes it a popular rootstock today. Other tolerant varieties include 'Thomas', 'Toro Canyon', 'Jovo', 'Martin Grande', 'Spencer' and 'G755'. 'Dusa®' (formerly 'Merensky 2') a new variety selected by Westfalia Technological Services (WTS) has surpassed the performance of 'Duke 7', especially under South African climatic conditions (Wolstenholme, 2003).

Although defense responses have been vigorously studied in model plant species, there is a vast amount of knowledge still to be discovered in non-model plants in order to comprehend the underlying defense mechanisms. Moreover, defense mechanisms can vary between different plant species and therefore each particular pathosystem of interest should be studied individually. Numerous studies have shown that salicylic acid (SA) is more involved in defense responses associated with biotrophic pathogens whereas jasmonic acid – ethylene (JA-ET) is more involved against necrotrophic pathogens and insects (Glazebrook, 2005; Kessler & Baldwin, 2002). Therefore, depending on the lifestyle of a pathogen, different defense mechanisms will be activated. *Phytophthora cinnamomi* is a hemibiotroph having both a biotrophic and necrotrophic phase (Hardham, 2007; Jackson & Taylor, 1996). This gives *P. cinnamomi* a great advantage; it is capable of switching to a necrotrophic phase when plant mechanisms are encountered that limit the spread of *P. cinnamomi*. In such a case, a defense mechanism like the HR would be ineffective against *P. cinnamomi* since it switches to the necrotrophic phase and uses the dead tissue as a nutrient source.

Traditionally, genetic resistance has been classified into two different types: qualitative and quantitative resistance. Qualitative resistance is mediated by *R*-genes and only provides short-lived resistance in the field as new virulent races of the pathogen rapidly overcome resistance encoded by single resistance genes. In contrast, quantitative resistance is controlled by multiple interacting genes that do not prevent infection, but slow down the development of the pathogen and hence, lasts longer. In the case of *P. cinnamomi* and

avocado, *R*-mediated resistance is unlikely to be the mechanism responsible for conferring tolerance to avocado rootstocks against *P. cinnamomi.* A multigenic trait is more likely as various levels of tolerance in avocado rootstocks against *P. cinnamomi* has been documented.

When *P. cinnamomi* infects avocado various defense responses are induced but few molecular studies have elucidated this interaction. In studies by Garcia Pineda *et al.* (2010) on the defense response in avocado roots against *P. cinnamomi*, a reactive oxygen species (ROS) burst as well as the involvement of catalase, epicathecin and nitric oxide (NO) were also highlighted. Muniz *et al.* (2012) used a proteomics approach to report on protein induction in response to infection of *P. cinnamomi* in roots of avocado. Proteins that were found to be involved in this interaction included isoflavone reductase, glutathione S-transferase, several abscisic acid-stress-ripening proteins (ASR), cinnamyl alcohol dehydrogenase, cinnamyl-CoA reductase, cysteine synthase and quinone reductase. These studies have barely begun to unravel how defense against *P. cinnamomi* is mediated within avocado and a comprehensive analysis of differentially expressed genes could contribute to a better understanding of the molecular processes involved in conferring tolerance to PRR. Knowledge of the genetic basis of this observed tolerance would not only contribute to the understanding of defense mechanisms but also aid in the development of superior avocado rootstocks.

This study elucidated the expression of known plant defense related genes in the response against *P. cinnamomi* in five avocado rootstocks that vary in PRR-tolerance. Expression of these genes was investigated over a time course following inoculation with *P. cinnamomi* using quantitative RT-PCR.

MATERIALS AND METHODS

Inoculation of avocado rootstocks with P. cinnamomi

Plant material

A hydroponic system previously used for banana (Van Den Berg *et al.*, 2007) was adapted for the avocado - *P. cinnamomi* pathosystem. Five avocado rootstocks were selected based on their tolerance level to *P. cinnamomi*. These rootstocks included R0.06 (highly tolerant); R0.09 (highly tolerant); R0.10 (tolerant - industry standard); R0.01 (tolerant) and R0.12 (least tolerant) and were obtained from WTS situated in Tzaneen, Limpopo, South Africa. The 9-month-old clonally propagated plantlets were removed from their bags and transplanted into 500 ml polystyrene cups filled with water to ensure easy access to the roots.

Inoculum preparation

A *P. cinnamomi* isolate provided by WTS was used as the inoculum source. Prior to inoculation, the identity of this isolate was confirmed by amplifying the species specific LPV3 fragment using LPV3-for (5' GTGCAGACTGTCGATGTG 3') and LPV3-rev (5' GAACCACAACAGGCACGT 3') primers (Kong *et al.*, 2003). Genomic DNA was extracted from mycelia growing on ½ PDA (10 g potato dextrose, 15 g agar) using PrepMan™ Ultra reagent (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions. The 20 µl PCR reaction contained 2.5 µl 10X PCR reaction buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 0.25 µM of each specific primer, 1 U *Taq* polymerase (Bioline Ltd., London) and 20 to 50 ng of template DNA. PCR cycling conditions were: an initial denaturation step at 94 ºC for 2 min, followed by 30 cycles of 94 ºC for 30 sec, 58.5 ºC for 30 sec and 72 ºC for 1 min and a final extension step at 72 ºC for 7 min. PCR products were separated and examined by electrophoresis on 2% agarose with GelRed (Biotium, Inc., California, USA) and visualized under UV light.

Mycelial suspension

The *P. cinnamomi* isolate was grown on ½ PDA at 25 ºC for four days, where-after a sterile broth (8 g D-glucose, 0.8 g Yeast extract, 800 ml distilled water, autoclaved at 121°C for 15 min) was inoculated with six agar blocks (10 mm x 5 mm) containing mycelia and shaken at 25 ºC at 150 rpm for 10 - 14 days. Mycelial balls were placed on Whatman filter paper and left to dry briefly. Mycelia (3.05 g) were mixed with one liter sterile H_2O and blended for a few minutes to macerate the mycelial pieces. The solution was finally mixed with 5 liters of H_2O and used for inoculation.

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 5 days. Small agar blocks (10 mm x 5 mm) containing mycelia were cut at 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 V8 juice with $CaCO_3$ in 1 L dH_2O) was added and left for at least three days at room temperature (ca. 25 ºC). The broth was removed and agar blocks containing mycelia were rinsed three times with H_2O after which 25 ml filtered stream water was added to each of the plates and incubated for 2 – 3 days at room temperature under UV light. Sporangia formation was monitored during this incubation step by using a Zeiss stemi 2000 stereo microscope (Carl Zeiss Ltd., Munchen, Germany). Once sufficient sporangia formation was observed, the cultures were cold shocked by placing them at 4 ºC for 45 min after which they were removed immediately and left at room temperature for one hour to stimulate the release of zoospores. The zoospore suspension was removed from the plates, pooled together and used for inoculation.

Inoculation and sample collection

Avocado roots were suspended in 500 ml polystyrene cups and inoculated with a mixture containing 50 ml macerated mycelia and 1.9 x 10^3 ml^{-1} zoospores. Control plants received sterile water instead of inoculum. Root tissue for RNA extraction was collected at 0, 3, 6, 12, 24, 48 and 72 hours post infection (hpi). Due to the constraint of limited avocado plants we did not include uninfected controls at each time point for this study. Root material from 3 - 5 plants per avocado rootstock was harvested per time point to form three biological samples representing either one or two avocado plants pooled together. Root tissue was immediately 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 ºC. After inoculation, plants were evaluated for root rot symptoms. Selected infected plants were transplanted to plastic bags containing perlite and kept for 6 weeks to confirm the development of PRR symptoms.

Gene expression profiling

RNA extraction and cDNA synthesis

RNA was extracted from root powder using a modification of the CTAB RNA extraction method developed by Chang *et al.* (1993) and stored at - 80 ºC. It is known that avocados have a high content of polysaccharides which seems to influence the quality of RNA, therefore the number of chloroform extractions and centrifugation steps was increased to four. Concentration of RNA was determined using a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, Inc., Montchanin, USA). RNA integrity was assessed under non-denaturing conditions as described. DNase treatment of RNA was performed by the addition of 1 µl RNase-free DNase (1 U/µl) (Fermentas Life Sciences, Hanover, USA), 1 µl 10X reaction buffer with $MgCl_2$, 1 µg RNA and DEPC treated water to a final volume of 9 µl. The mixture was incubated at 37 ºC for 30 min followed by the addition of 1 µl 25 mM EDTA and

incubated at 65 ºC for 10 min. DNase-treated RNA was column purified using the RNeasy® MiniElute™ Cleanup kit (Qiagen, Valencia, USA) according to the manufacturer's instructions.

First strand cDNA synthesis was carried out in a total volume of 5 µl that consisted of 0.5 µg random hexamers (Invitrogen Life Sciences, USA, California), 1 µg RNA from the previous step and RNase free water. The mixture was incubated at 70 ºC for 5 min and then chilled on ice for 5 min, followed by the addition of 1 µl RNase inhibitor (40 U/µl) (Fermentas, Ontario, Canada), 1 µl 10 mM dNTPs (Fermentas, Ontario, Canada), 2.4 µl $MgCl_2$, 4 µl 5 X ImProm-II™ reaction buffer and 1 µl ImProm-II™ Reverse Transcriptase (Promega Corporation, Madison, USA). Finally the mixture was incubated at 25 ºC for 10 min followed by 42 ºC for 60 min and 70 ºC for 10 min.

The cDNA was analyzed for genomic DNA contamination by PCR, using gene specific primers F3H-for (5' TCTGATTTCGGAGATGACTCGC 3') and F3H-rev (5' TGTAGACTTGGGCCACCTCTTT 3'), that flanked an intron of the *Flavanone 3-hydroxylase* (*F3H*) gene. PCR amplifications were carried out using first strand cDNA as the template. The PCR reaction mixture of 20 µl final volume contained 2.5 µl 10X PCR reaction buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 0.25 µM of each specific primer, 1 U *Taq* polymerase (Bioline Ltd., London) and 1 µl cDNA and water. Amplifications were performed in an Eppendorf MasterCycler® gradient (Eppendorf, Hamburg, Germany) under the following conditions: an initial denaturation at 94 ºC for 2 min, followed by 30 cycles of 94 ºC for 30 sec, 55 ºC for 30 sec and 72 ºC for 1 min. Final extension was carried out at 72 ºC for 7 min. PCR products were visualized as before.

Real- time RT-PCR primer design

The expression of seven avocado defense-related genes namely *phenylalanine ammonia-lyase (PAL), lipoxygenase (LOX), pathogenesis-related protein* 5 (*PR-5*), *endochitinase*,

gluthathionine S-transferase (GTH), heat shock protein (HSP) and *metallothionein* were investigated. These genes were selected based on their role in plant defense against pathogens. *Actin,* 18S rRNA and *glyceraldehyde 3-phosphate dehydrogenase (GADPH)* were used as endogenous controls. Primers for these genes were designed from sequences obtained from the NCBI database by using Primer 3 software (Rozen & Skaletsky, 2000) and synthesized by either Operon Biotechnologies GmbH (Cologne, Germany) or Inqaba Biotec (Hatfield, South Africa) (Table 1).

Primer pairs were tested for successful amplification of target genes with cDNA in a conventional PCR assay. The PCR reactions were carried out in a total volume of 20 µl containing contained 2.5 µl 10X PCR reaction buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 0.25 µM of each specific primer, 1 U *Taq* polymerase (Bioline Ltd., London) and 1 µl of cDNA. The cycling conditions were 95 ºC for 3 min, followed by 44 cycles at 94 ºC for 30 sec, 59 ºC for 30 sec, and 72 ºC for 30 sec, with a final elongation step at 72 ºC for 10 min. PCR products were analyzed as before.

RT-PCR optimization

A dilution series and standard curve were generated for each gene to examine the linearity of amplification over a dynamic range. Quantitative RT-PCR was performed using the Bio-rad® CFX 96 instrument. Serial dilutions (1:10, 1:25, 1:50, 1:100 and 1:1000) were performed using 5 µl of diluted cDNA from a mixture of all treatment samples inoculated with the pathogen to calculate the standard regression curves. Each reaction was performed in triplicate. A 20-µl reaction for PCR amplification contained 10 µl Sensimix™ SYBR No-ROX Kit (Bioline Ltd, London, UK), 1 µl of each of the forward and reverse primers (10 µM), 5 µl diluted cDNA template and 3 µl PCR grade water (Roche Diagnostics). The cycling conditions were as follows: pre-incubation for 10 min at 95 °C (hot start) followed by 44 cycles, each consisting of 10 sec denaturing at 95 °C, 10 sec annealing at 59 °C, 10 sec primer extension at 72 °C, and data acquisition at 95 °C.

Three endogenous control genes were evaluated for their efficiency as reference genes by conducting a real-time PCR experiment using known amounts of avocado cDNA. Expression of these genes was analyzed across different time points after infection in order to assess whether they were expressed constitutively in all samples for use for normalization of the qRT-PCR data. Data were analyzed using CFX Manager Software to determine which genes were most suitable for use in gene expression studies.

Gene expression analysis using quantitative real-time RT-PCR

Quantitative RT-PCR was performed as described previously. Control treatments contained water as template. All PCR reactions were performed in triplicate on each of the three independent biological replications. The cycling conditions were the same as described for the optimization conditions and each reaction contained 5 µl of template dilution. Melting curve analysis of the qRT-PCR products was performed to confirm that the individual qRT-PCR signals corresponded to a single homogenous cDNA product.

Data analyses

Standard regression curves were calculated from amplification data of 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) (Ginzinger, 2002). The resulting crossing point (CP) values for each input amount of template were plotted as a function of the log [10] concentration of input amounts and a linear trend-line was imposed on the data. R^2 is the proportion of variability that the data set is accounted for by a statistical model, thus, $R^2 = 1$ indicates that the fitted model explains all variability in y, while $R^2 = 0$ indicates no linear relationship between the response variable and regressor. Statistical significance from the qRT-PCR data was analyzed by one way ANOVA followed by a LSMeans Differences Student's t-test using the software package of JMP '9 (SAS Institute, Inc., Cary, NC). In all cases significance was evaluated at $P < 0.05$.

RESULTS

Confirmation of P. cinnamomi and symptom evaluation of avocado rootstocks

The identity of the *P. cinnamomi* isolate was confirmed. The amplification of a region of the *LPV3* gene from DNA yielded the expected amplicon size of 450 bp (Fig. 1). Avocado plants (five rootstocks) infected with *P. cinnamomi* developed typical root rot symptoms six weeks after infection. These symptoms included necrotic lesions that developed on the roots and lower stem and some of the feeder roots were black and brittle. Plants showed yellowing and wilting of leaves and in severe cases, leaf abscission and death. The highly tolerant R0.06 appeared the healthiest when compared to the least tolerant rootstock (R0.12) which showed severe symptoms. The pathogen was re-isolated and Koch's postulates were proven.

RNA extraction and cDNA synthesis

Avocado root RNA samples were of high quality (Fig. 2.A) and the absence of contaminating genomic DNA was confirmed for all cDNA samples (Fig. 2.B). The amplification of a region of the *F3H* gene from cDNA yielded the expected 241 bp RNA-derived product, which was clearly distinguishable from the 1221 bp genomic DNA-derived, intron-containing product (Fig. 2.B).

Generation, optimization and analysis of endogenous genes for qRT-PCR

The three primer pairs that respectively amplified a portion of *Actin*, 18S rRNA and *NADPGH* were evaluated for their suitability to serve as endogenous controls. Two of the three primer pairs (*Actin* and 18S rRNA) successfully amplified a PCR product of the expected size. The primer pair for *NADPGH* displayed multiple bands and was therefore discarded. *Actin* and 18S rRNA showed C_T (threshold cycle for each trace) differences less than 1.5 between different treatments and consistently exhibited M values (reference gene stability value) less than 0.50. Therefore primer pairs amplifying these two gene regions were selected as

endogenous controls for this study (Table 2). "Gene stability is expressed as an M value, which is inversely proportional to the variation in expression for a given gene (Vandesompele *et al.*, 2002)." The lower the M-value, the more stably expressed the reference gene is.

Real- time RT-PCR Primer design

All primer pairs designed for defense-related genes *phenylalanine ammonia-lyase (PAL), lipoxygenase (LOX),* pathogenesis-related protein 5 (*PR-5*), *endochitinase*, *gluthathionine S-transferase (GTH), heat shock protein (HSP)* and *metallothionein* produced single products between 75–150 bp as expected. The optimal annealing temperature for all the primer pairs was 59 °C (Table 1). The standard curves provided insight into the PCR efficiency for each particular primer set (Fig. 3).

Gene expression profiling

Basal (0 hpi) and early expression (3, 6 and 12 hpi) of *PR-5* was very low and similar in tolerant and less tolerant avocado rootstocks (Fig. 4.A). The first significant response after *P. cinnamomi* infection occurred at 24 hpi in the moderately tolerant R0.10. By 48 hpi all rootstocks (except R0.10) had a significant increase in *PR-5.* The least tolerant R0.12 and highly tolerant R0.06 had the highest expression at 48 hpi. By 72 hpi *PR-5* expression in all rootstocks continued to increase significantly. The highest expression level of *PR-5* was observed at 72 hpi for all rootstocks. R0.06 and R0.09 had a 42- and 24-fold increase respectively compared to the uninfected roots (0 hpi) this did not significantly differ from the least tolerant R0.12 which also responded with a 19 fold increase (Fig. 4.A).

The basal expression of *PAL* was significantly higher in the least tolerant R0.12 compared to all rootstocks except R0.09 (Fig. 4.B). By 3 hpi all rootstocks (except R0.01) showed a decrease in *PAL* levels (although not significant). A significant increase in *PAL* was observed in the highly tolerant R0.09 at 6 hpi. *PAL* levels in the less tolerant R0.12 and moderately

tolerant R0.10 continued to decrease significantly at 6 hpi whereas R0.06 and R0.09 only decreased at 12 hpi. By 24 hpi R0.06 and R0.09 once again had increased significantly but levels of *PAL* between the different rootstocks were similar (Fig. 4.B). At 48 hpi *PAL* expression in R0.12 and R0.10 was significantly lower than in the other rootstocks and remained low at 72 hpi. The levels in all other rootstocks remained similar at 48 hpi when compared to levels at 24 hpi. By 72 hpi the highly tolerant R0.09 also exhibited a significant decrease in *PAL* levels (Fig. 4.B). Noteworthy is that expression levels of *PAL* in the less tolerant R0.12 continued to decrease over the entire time course.

The constitutive expression of *LOX* was significantly higher in the highly tolerant R0.09 when compared to the other rootstocks. *LOX* levels in R0.09 decreased significantly 3 hpi (Fig. 4.C). The levels remained similar in all rootstocks 6 and 12 hpi. By 24 hpi R0.10 and R0.09 showed significantly higher levels when compared to 12 hpi and when compared to other rootstocks at 24 hpi. *LOX* levels in R0.01 and the less tolerant R0.12 increased significantly from 24 hpi to 48 hpi. At 72 hpi R0.06 and R0.01 showed a significant increase in *LOX*, with gene expression in R0.01 being the highest across all time points and rootstocks (Fig. 4.C). R0.01 had a 8 fold increase at 72 hpi compared to 0 hpi.

Basal levels of *endochitinase* transcripts were low in all rootstocks and significantly lower in highly tolerant R0.06 and moderate tolerant R0.01 when compared to less tolerant R0.12. Levels remained similar at 3, 6 and 12 hpi. The industry standard (Duke 7) R0.10 exhibited a response at 12 hpi with a significant increase (Fig. 4.D). The highly tolerant rootstocks R0.06 and R0.09 as well as R0.01 showed significant increases in *endochitinase* expression at 24 hpi, compared to the less tolerant R0.12 that remained unchanged when compared to the response at 12 hpi (Fig. 4.D). By 48 hpi levels of *endochitinase* continued to increase in R0.06 and R0.09. Highly tolerant R0.09 had the highest expression of *endochitinase* at 48 hpi, by this time R0.12, R0.10 and R0.01 also showed significant increases in *endochitinase* expression (Fig. 4.D). At 72 hpi *endochitinase* expression in R0.06, R0.01 and R0.1

continued to increase compared to a decrease in the less tolerant R0.12 whereas *endochitinase* expression in R0.09 remained high. *Endochitinase* gene expression in R0.01 was significantly the highest at 72 hpi (Fig. 4.D).

Metallothionein expression was significantly higher in the highly tolerant R0.09 when compared to the less tolerant R0.12 and R0.10 before infection with *P. cinnamomi*. The only significant response after pathogen infection was an increase in *metallothionein* in the less tolerant R0.12 at 3 hpi, followed by a sharp decrease at 6 hpi (Fig. 4.E). R0.06 and R0.09 had significant increased expression of *metallothionein* compared to the less tolerant R0.12 at 6 hpi and 12 hpi respectively. At 12 hpi R0.10 also exhibited a significant increase of *metallothionein* expression. Noteworthy is the constant high level of *metallothionein* in R0.09 over the first 24 hours. By 24 hpi the highly tolerant R0.06 had a expression level of 1.2 resulting in a significant higher level compared to the less tolerant R0.12 (Fig. 4.E). By 48 hpi all rootstocks except the highly tolerant R0.06 and R0.09 showed significant decreases in *metallothionein*. Furthermore, R0.06 and R0.09 continued to have higher expression of *metallothionein* compared to the less tolerant R0.12. By 72 hpi *metallothionein* expression in all rootstocks had declined, however the highly tolerant R0.06 remained significantly higher compared to all other rootstocks (Fig. 4.E).

Basal expression of *GTH* did not differ significantly between R0.12, R0.10, R0.06 and R0.09. Levels remained unchanged by 3 and 6 hpi; except in the case of R0.10 which showed a significant decrease at 6 hpi (Fig. 4.F). By 12 hpi levels of *GTH* in R0.10 increased significantly. At 24 hpi the less tolerant R0.12 and tolerant R0.01 exhibited significant increases in *GTH* levels. By 48 hpi all rootstocks except highly tolerant R0.06 and R0.09 displayed significant decreases in *GTH*. At 72 hpi the less tolerant R0.12 had the lowest *GTH* of all rootstocks (Fig. 4.F).

The constitutive expression of *HSP* was significantly the lowest in the less tolerant R0.12 rootstock. By 3 hpi *HSP* expression in R0.12 increased significantly followed by a significant

decrease at 6 hpi but remained similar in all other rootstocks (Fig. 4.G). Levels of *HSP* in the less tolerant R0.12 remained significantly the lowest at 12 hpi, while R0.10 had a significant increase in *HSP* at the same time point. By 24 hpi and 48 hpi expression of *HSP* in all rootstocks remained similar, except R0.10 that decreased significantly at 24 hpi (Fig. 4.G). All rootstocks except R0.01 and R0.06 had a significant increase in *HSP* expression at 72 hpi.

DISCUSSION

This is the first study of its kind aimed at unraveling the regulation of a set of seven defense associated genes during the first 72 hours post infection in five avocado rootstocks with different levels of tolerance to PRR. A comprehensive understanding of the molecular mechanisms involved during the tolerant host response will enable the identification of defense marker genes that in turn will aid in the selection of avocado rootstocks with enhanced PRR-tolerance. Phenotypic PRR tolerance data for R0.06 and R0.09 has been collected over at least a decade by WTS and both these rootstocks have consistently shown high levels of tolerance in the field under various climatic conditions. Additionally to PRR-tolerance both rootstocks have produced high yields when grafted with Hass. In South Africa, 'Dusa®' (R0.09) has replaced 'Duke 7' (R0.10) as the preferred rootstock for commercial avocado production. Rootstock R0.12 has the lowest level of tolerance of all the rootstocks used in this study. However, it possesses some tolerant attributes and was initially selected in greenhouse trails as a promising candidate but failed during field trails. While there were no uninfected control plants for each time point, due to a limited number of plants, the data generated from this study still provides insights into the role of the selected genes in the defense response as well as in conferring tolerance to PRR.

PR-5 was significantly up-regulated at 24, 48 and 72 hpi in all rootstock varieties infected with *P. cinnamomi*. The slow but continuous up-regulation of *PR-5* in all avocado rootstocks

suggests that the gene may be an important early defense strategy against the biotrophic *P. cinnamomi.* PR proteins are defined as localized proteins (intra- and extracellular) that accumulate in distant plant tissue after pathogen attack (Bowles, 1990). For many years PR proteins have been correlated with the development of systemic acquired resistance (SAR) (Durrant & Dong, 2004). Although they are widely used as markers for SAR, their exact roles have not yet been identified. It has been shown that the induction of *PR-5* requires SA signalling and that SA signalling is more associated with defense against biotrophs (Glazebrook, 2005; Kessler & Baldwin, 2002). The fact that *PR-5* was also up-regulated in the less tolerant rootstock (R0.12) was expected as previous studies showed that R0.12 possessed slight tolerance against *P. cinnamomi* albeit weak. From this study it seems that *PR-5* expression cannot be correlated with phenotypic PRR tolerance, but the gene is induced in response to *P. cinnamomi* infection.

Expression analysis of *PAL* after infection with *P. cinnamomi* showed that the less tolerant R0.12 and R0.10 rootstocks showed strong down regulation of *PAL* expression. It can be hypothesized that this down-regulation of *PAL* might be necessary for the establishment and development of *P. cinnamomi*. Upon recognition of *P. cinnamomi* by avocado, a series of signalling pathways are activated. Among theses, the phenylpropanoid pathway plays an extremely important role in secondary plant metabolism by producing numerous phenolic propanoids such as phenolic acids and flavonoids that have vital structural and defense related functions (Sgarbi *et al.*, 2003; Solecka & Kacperska, 2003). Phenylalanine ammonia-lyase is one of the fundamental enzymes in the phenylpropanoid pathway. Therefore it is possible that more phenolic compounds are produced in the highly tolerant rootstocks to combat *P. cinnamomi* growth whereas the down regulation in the less tolerant rootstocks implicates much less phenolic compounds being produced. In pepper (*Capsicum annuum* L.) an increase in phenolic acids reduced lesion length and invasion upon infection by *Phytophthora capsici* whereas susceptible cultivars of *C. annuum* produced lower amounts of phenolic acids and lesion lengths were not reduced (Candela *et al.*, 1995).

Inoculation of avocado plants resulted in significant increased levels of *LOX* gene expression for R0.09 and R0.10 at 24 hours, as it has also been reported to occur in other plant pathogen interactions (Koch *et al.*, 1992; Melan *et al.*, 1993; Peng *et al.*, 1994). Lipoxygenases (EC 1.13.1 1.12) are a family of enzymes that catalyze the dioxygenation of polyunsaturated fatty acids (PUFAs) in lipids. The role of *LOX* in defense against pathogens is connected to the production of compounds that are involved in signalling (Creelman & Mullet, 1997), antimicrobial activity (Weber *et al.*, 1999), and HR development (Rustérucci *et al.*, 1999). The fact that R0.12 remained unchanged during the first 24 hours indicates a delayed response and therefore subsequent failure to restrict the pathogen by not allowing signalling of other defense responses or by a lack of antimicrobial activity. Tomato plants infected with *Phytophthora infestans* have been shown to display fungitoxic activities by producing linolenic acid via lipoxygenase (Kato *et al.*, 1983).

An increase in expression of *endochitinase* became evident at 24 hpi in all rootstocks with R0.12 being the exception. R0.12 was the only rootstock that did not show any significant changes during the first 24 hpi. This suggests that this delay of 24 hours allows establishment of *P. cinnamomi* in the less tolerant rootstock when compared to other rootstocks that showed significant up-regulation at 24 hpi. *Endochitinases* play a role in plant defense by attacking structural chitin present in the cell wall of fungi (Sela-Buurlage *et al.*, 1993). Unlike fungal cell walls, oomycete cell walls are mainly composed of cellulosic compounds and glucans but have been found to contain limited amounts of chitin (Erwin *et al.*, 1983; Erwin & Ribeiro, 1996). Consequently, limited studies have been conducted to investigate the role of *endochitinase* upon infection with oomycetes such as *Phytophthora* spp. From the present study it is clear that *endochitinase* was activated upon infection and at 72 hpi it was highly up-regulated compared to the uninfected in all rootstock varieties. Similar results were obtained by a study conducted by Mishra *et al.* (2010) who demonstrated by use of Northern blot analysis that transcripts of *endochitinase* were highly up-regulated

reaching the highest expression 36 hr after *Phytophthora colocasiae* elicitor treatment of taro cells.

Both *glutathione-S-transferase* and *metallothionein* are known to function as ROS scavengers. Antioxidant genes like *glutathione-S-transferase and metallothionein* displayed high constitutive expression particularly in the more tolerant avocado rootstocks. Plants rely on multiple enzymes to scavenge reactive oxygen species to restore balance of toxic molecules (Morita *et al.*, 1999). Quite to the contrary, it was unexpected that *GTH* and *metallothionein* were not significantly up-regulated in avocado roots after *P. cinnamomi* treatment since scavenger molecules are known to be highly expressed during pathogenic infection to remove reactive oxygen species as the presence of ROS species can be indicative of an HR activity. The highly tolerant R0.09 and R0.06 displayed no significant responses for *metallothionein* and *glutathione-S-transferase* except at 72 hpi where they were significantly down-regulated and for *metallothionein* at 24 hpi in R0.09. GTH has been shown to be up-regulated in leaf tissue but down-regulated in the root tissue of *Coffea arabica L* upon BTH treatment to mimic plant disease which supports the down-regulation that occurred at 72 hpi in this study. Based on our present results both *glutathione-S-transferase* and *metallothionein* does not seem to be involved in conferring tolerance in the defense response against *P. cinnamomi as* the expression patterns of these genes in the highly tolerant rootstocks were not altered by pathogen infection. It is however possible that the tolerant cultivars maintained an equilibrium that is sufficient to protect from a ROS burst.

Expression of *HSP* varied among the rootstocks. The less tolerant rootstock R0.12 had the lowest levels of *HSP* across early time points (0, 6, 12 and 48 hpi), whereas the tolerant rootstocks maintained moderate levels of expression compared to other rootstocks. Muniz *et al.* (2012) were the first group to report on protein induction using a proteomics approach in response to *P. cinnamomi* infection of avocado roots. After identifying *HSP* as an induced protein upon infection, gene expression studies using northern blot analysis were conducted

where it was shown that *HSP* was down-regulated, decreasing steadily up until 6 hpi. Heat shock proteins (HSPs) are expressed in response to various stresses such as cold, salt, drought and oxidative stress (Boston *et al.*, 1996; Vierling, 1991; Waters *et al.*, 1996). Kanzaki *et al.* (2003) showed that silencing of *HSPs* in *Nicothiana benthamiana* compromised resistance to *Phytophthora infestans* when compared to wild type plants. A typical HR developed in wild type plants, whereas silenced plants did not produce a HR after infection with INF1 elicitor, indicating the importance of HSPs in the development of defense responses such as HR. The high up regulation at 72 hpi indicate an active role in the defense response against *P. cinnamomi* infection.

CONCLUSION

The focus of this study was to obtain a overview of the expression patterns of selected avocado genes during *P. cinnamomi* infection, with the intention of further understanding the mechanism of tolerance observed. Since differences in gene expression are responsible for morphological and phenotypical differences, gene expression profiles of avocado over a time period, infected with *P. cinnamomi* provided evidence of genes involved in the tolerance and serve as a basis for investigating plant–pathogen interactions and gene function.

The genes investigated in this study indicated that they are involved in the defense response although their specific role in inferring tolerance observed against *P. cinnamomi* was unclear as rootstocks shared similar expression patterns for many genes investigated. The only genes to show differences in expression between tolerant rootstocks and less tolerant rootstocks were *LOX* and *PAL*. It is highly likely that partial resistance or tolerance to some pathogens, such as *P. cinnamomi*, is much more complex and involves the interaction of many genes at various levels and is different from those associated with *R*-gene mediated resistance. Identifying the genes responsible for the tolerance observed in avocado rootstocks to *P. cinnamomi* remains a challenge. Further studies are needed to understand

how tolerance against *P. cinnamomi* is governed in avocado; this will help to advance our understanding of quantitative disease resistance in plants as well as aid to develop markers for marker-assisted breeding for the development of resistant avocado rootstocks.

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FIGURES AND TABLES

Table 1. Primer sequences of defense-related genes studied in avocado roots by quantitative reverse PCR following infection by *Phytophthora cinnamomi.*

Target gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Product size (bp)
PAL	AATCTTGGAAGCAATCAC	CAGCAATGTAGGATAAGG	110
LOX	GTTAATCCAGTTATCATCAG	TAGGTTCTTCTCAATGTG	117
HSP	AGAGGAGGAAGAGGAATG	TCAATGTTCTCAGGCAATG	75
PR-5	TAATGAGTATTGTTGCGATAAAGG	TGGGCATCTGTCTTTGAAG	76
METAL	AAGTGGCTGTGGAGGATG	CATAATCAAGGTCTCAGAGGTG	79
GTH	GCGATTACAAACTACATAAG	ATCCACACTGCTACTATC	95
Actin	CTCACGGATGCTCTAATG	CTTACAATTTCAGGCTCAG	77
18S rRNA	GTTACTTTAGGACTCCGCC	TTCCTTTAAGTTTCAGCCTTG	90
GADPH	CAAAGCTGCAATCAAGGAGGA	ACCTGCTGTCACCCACCAAGT	101
Endochitinase	ATCACCAACATCATCAAC	CTCTTGTAGAAGCCAATG	83

Table 2. Evaluation of uniform expression of candidate reference genes *(Actin, GADPH* and 18S rRNA) over different treatments indicating the M-value (reference gene stability value) and coefficient variance for each gene. An M-value of 0.5 and lower is regarded as suitable for use as a reference gene.

Target gene	Coefficient Variance	M-Value
Actin	0.3988	0.1543
18S rRNA	0.5472	0.2508
GADPH	2.2475	0.98

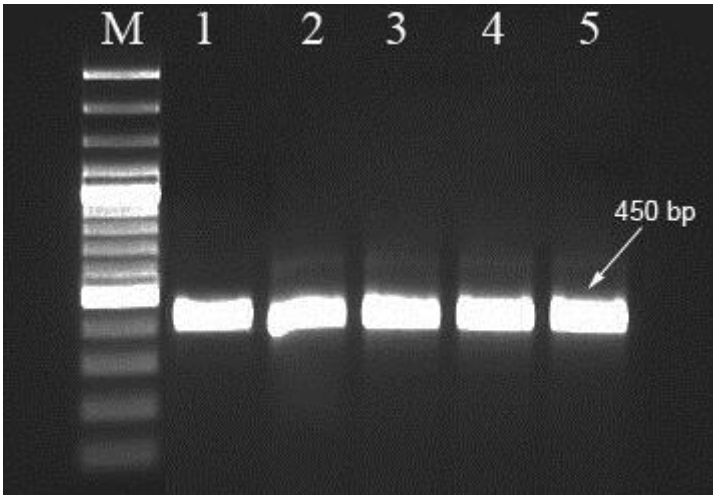


Figure 1. Agarose gel electrophoresis of PCR products for identification of *Phytophthora cinnamomi.* using *LPV3* species specific primers M: 100-bp molecular weight standard; Lane 1 – 5: 450 bp product from DNA that was isolated from *P. cinnamomi* culture obtained from WTS.

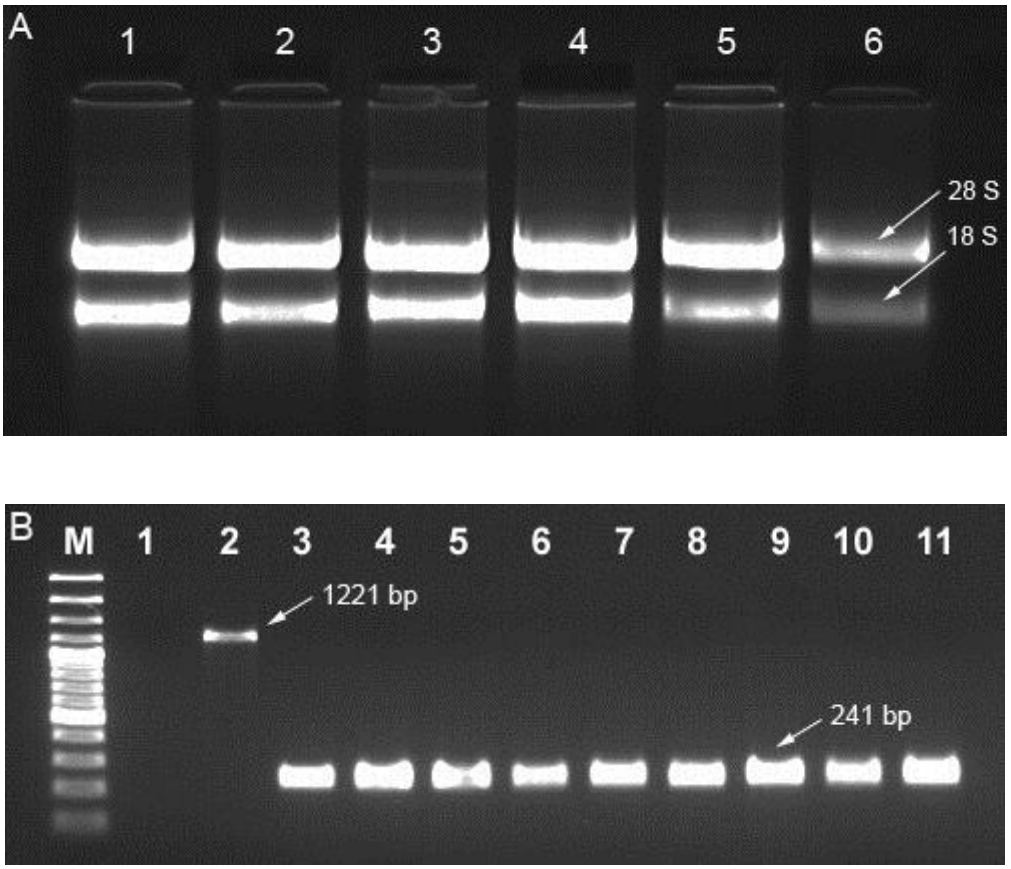


Figure 2. **(A)** Total RNA extracted from avocado roots infected with *Phytophthora cinnamomi* as revealed by non-denaturing gel electrophoresis, with two distinct the 28S and 18S rRNA bands. **(B)** PCR products with *F3H*-based control primers for monitoring genomic DNA contamination of avocado cDNA. M: 100-bp molecular weight standard; 1 - Negative control; 2 - Genomic DNA control - 1221 bp, 3 to 11 - 241 bp PCR products from avocado root cDNA.

STANDARD CURVES

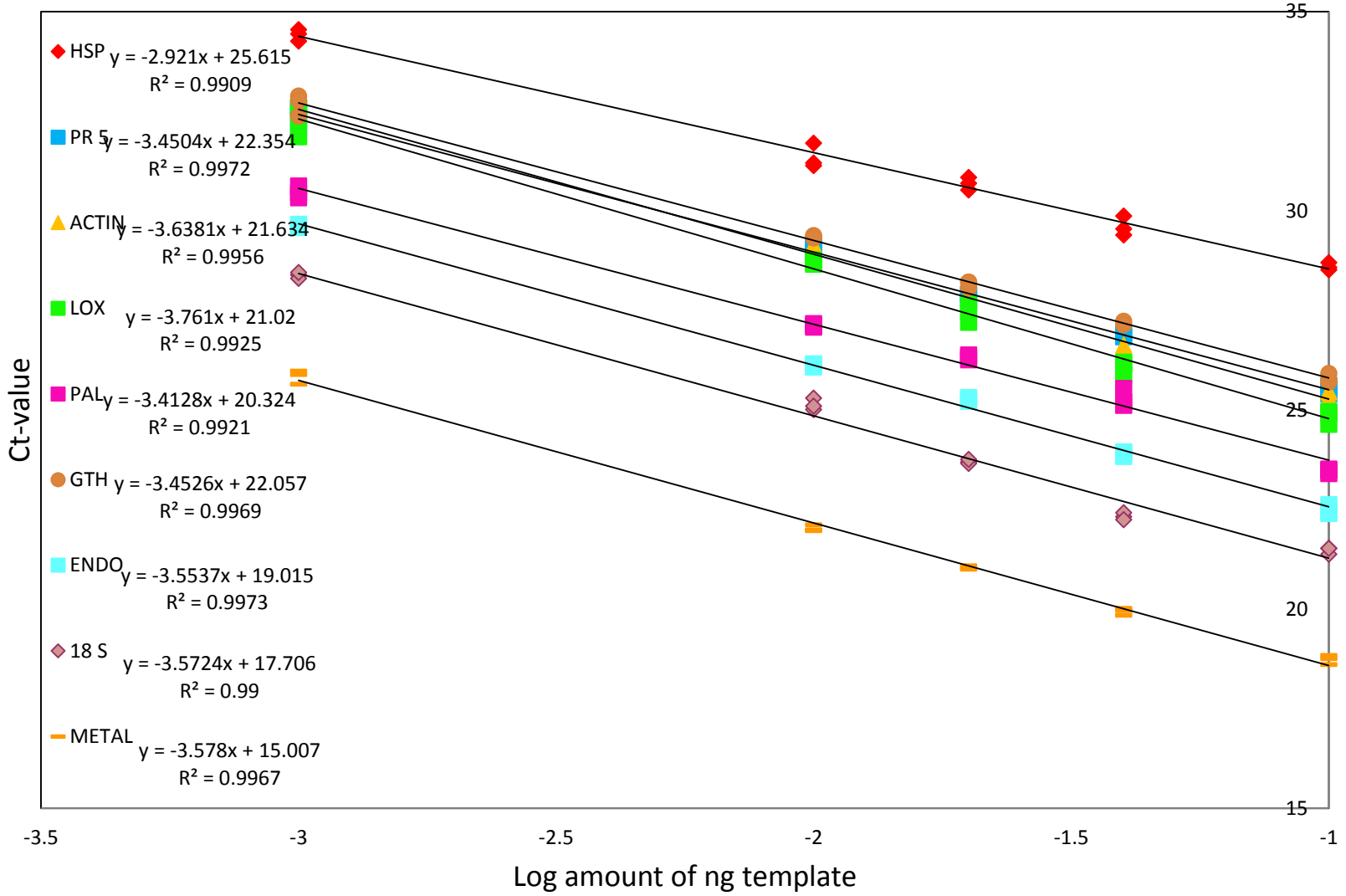
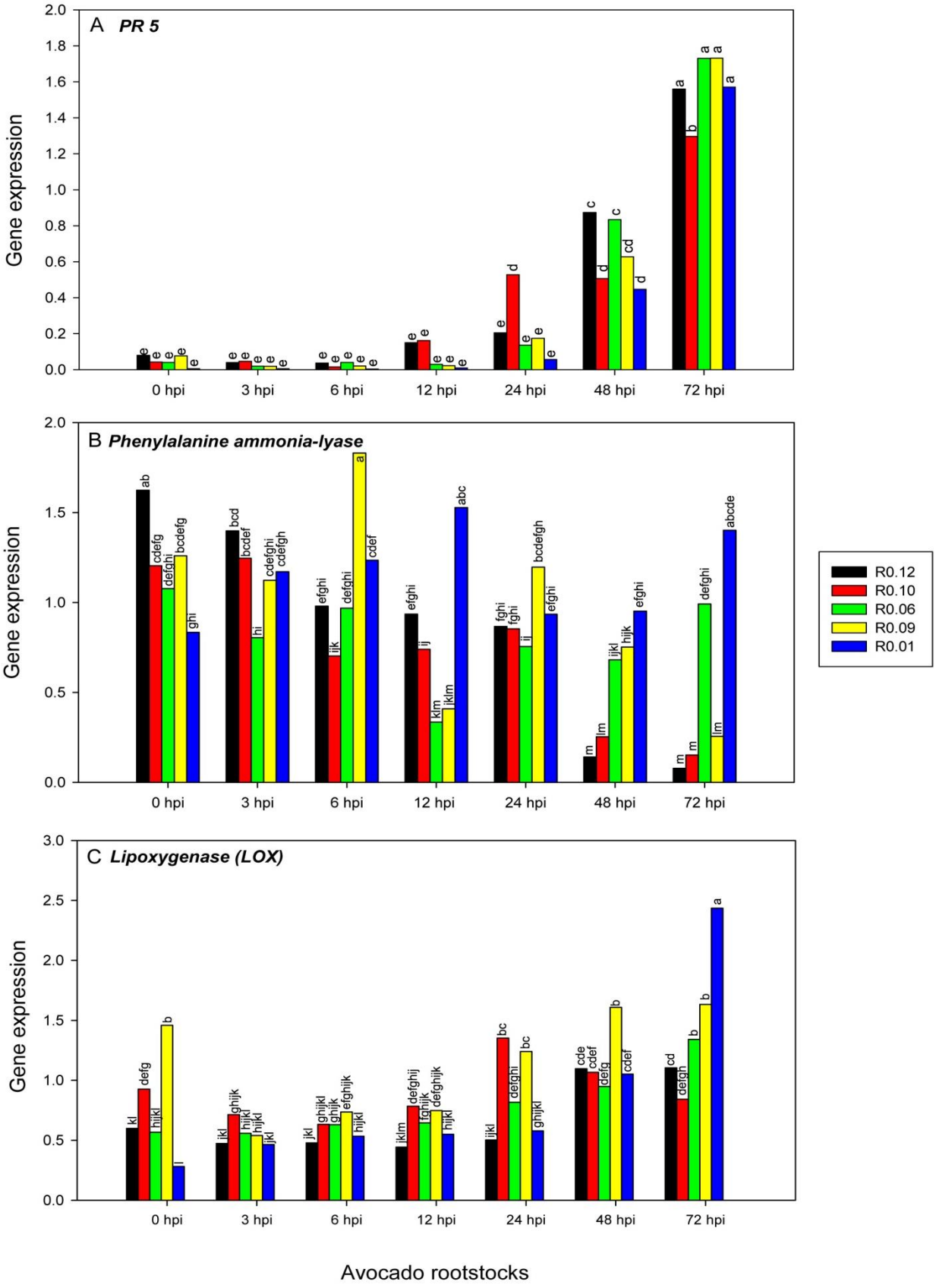
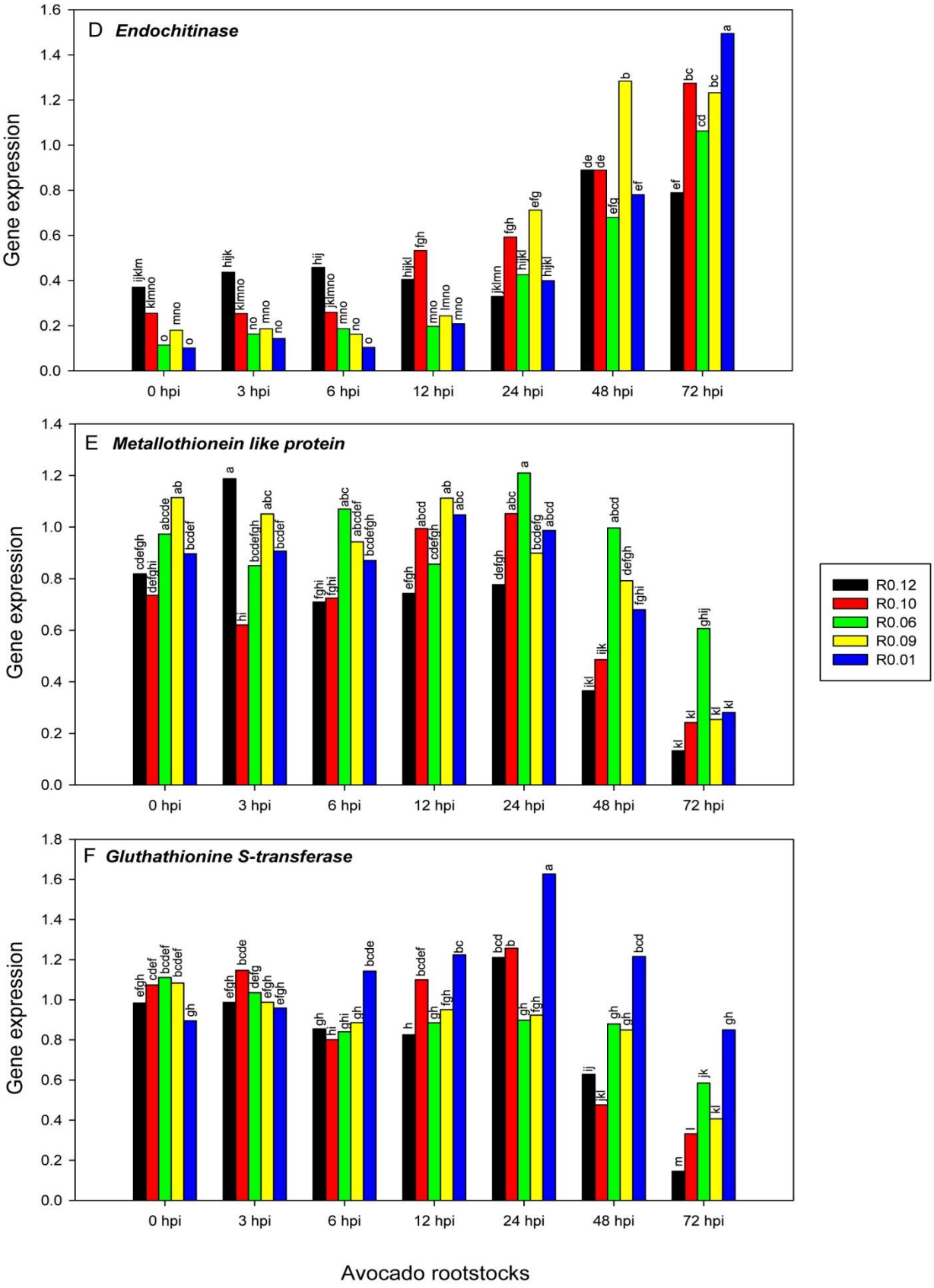


Figure 3. Standard regression curve plots. A dilution series of a mixture of cDNA spanning five orders of magnitude (1:10, 1:25, 1:50, 1:100, 1:1000) was used to generate standard curves for each separate primer pair: r18S (A), *Actin* (B), *lipoxygenase* (C), *phenylalanine ammonia lyase* (D), *PR5* (E), *HSP* (F), *gluthathionine S-transferase* (G), *metallothionine like protein* (H) and *endochitinase* (I). The resulting crossing point (CP) values for each input amount of template were plotted as a function of the log [10] concentration of input amounts and a linear trend-line was imposed on the the data. R^2 is the proportion of variability that the data set is accounted for by a statistical model, thus, $R^2 = 1$ indicates that the fitted model explains all variability in y, while $R^2 = 0$ indicates no linear relationship between the response variable and regressors.





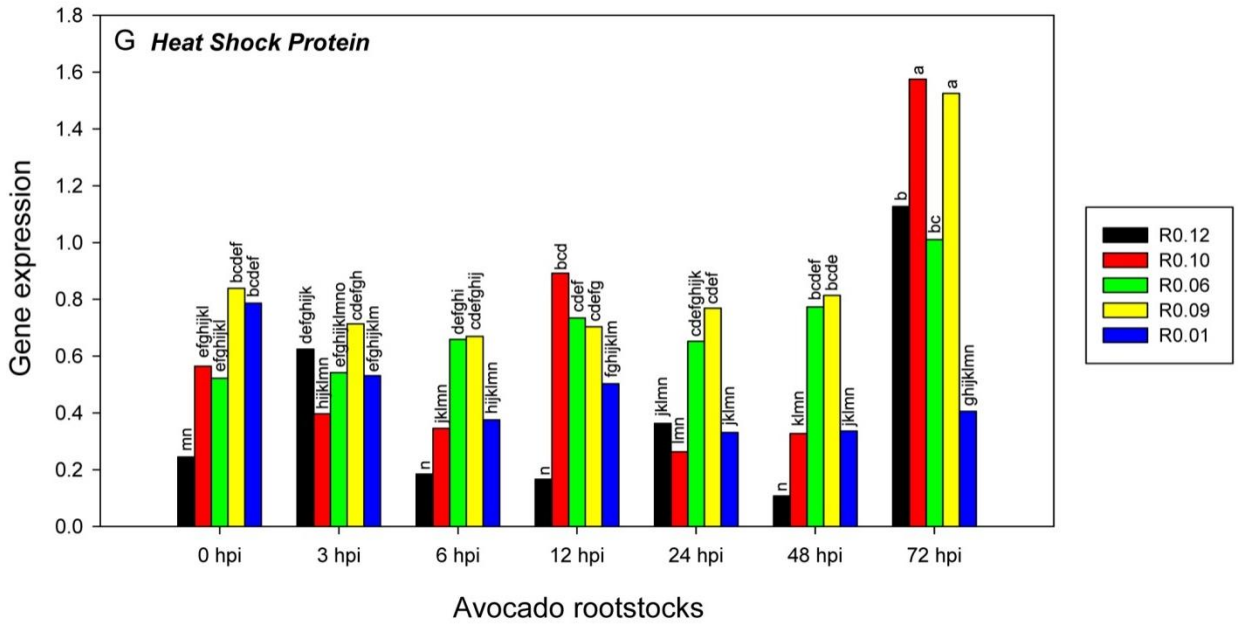


Figure 4. Relative expression levels of defense associated genes in avocado rootstocks infected with *Phytophthora cinnamomi* at 0, 3, 6, 12, 24, 48 and 72 hpi. *PR 5* (A), *phenylalanine ammonia lyase PAL* (B), and *lipoxygenase LOX* (C) *endochitinase ENDO (D), metallothionine like protein METAL (E), gluthathionine S-transferase GTH (F), heat shock protein HSP (G)*. Expression ratios were determined by quantitative RT-PCR. Data sets were analyzed using ANOVA and LSMeans Students t-test. Bars represented with the same letter are not significantly different at *P*>0.05. The X axis represents the avocado rootstocks {R0.12 - least tolerant (black), R0.10 - tolerant industry standard (red), R0.06 - highly tolerant (green), R0.09 - highly tolerant (yellow) and R0.01 – tolerant (blue)} infected over time and the Y axis represents the relative gene expression level.

CHAPTER 4

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

ABSTRACT

Phytophthora cinnamomi (Pc) is the causal agent of Phytophthora root rot in avocado (*Persea americana*), one of the most important diseases causing severe economic losses to the avocado industry globally. To date, no Pc-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 was used to enhance the sensitivity of detection of pathogen DNA isolated from plant tissues. Roots samples were collected at 0, 3, 7, 14 and 21 days after infection with *P. cinnamomi* and used for pathogen quantification. Results showed that nested primers developed in this study were specific and sensitive and could detect *P. cinnamomi* in root tissues, even in the latent period, where the first symptoms of the disease had not yet appeared. Results indicated that the amount of *P. cinnamomi* quantified in roots was significantly higher in the less tolerant R0.12 plants when compared to the highly tolerant R0.09 plants at all time points except for day three. This study has confirmed the known status of disease tolerance of these avocado rootstocks in a quantitative manner. This assay provides a reliable molecular tool to assist with industry breeding programs for the selection of resistant avocado rootstock varieties against Phytophthora root rot.

INTRODUCTION

Phytophthora root rot (PRR) is an important disease of avocado caused by the oomycete *Phytophthora cinnamomi* Rands. In the agricultural sector, PRR of avocado has resulted in severe crop losses in most avocado producing countries around the globe. The first symptoms of PRR infection become visible on the root system and only at a later stage when infection is severe will aboveground symptoms become apparent. Infected plants can appear asymptomatic for an extended period of time, which could lead to the underestimation of disease and inappropriate control measures. Thus far pathogen quantification of *P. cinnamomi* in woody hosts such as the avocado has not yet been conducted.

Traditionally, *P. cinnamomi* detection is based on the visual assessment of symptoms and the microscopic identification of the pathogen after culturing on selective media. However, pathogen quantification by these methods is not entirely reliable as a calculation of pathogen biomass by microscopy is laborious and results can differ greatly between investigators (Nicolaisen *et al.*, 2009). Chemical methods such as fatty acid ergosterol and carbohydrate chitin are widely used whereby the amount of a specific bio-molecule either present within pathogen cells or released into the environment is determined (Gessner & Newell, 2002; Wallander *et al.*, 2001). Although widely implemented and not as laborious as microscopy, these methods lack specificity and become problematic when processing field samples where the minimal sample size required is relatively high.

In recent years, several techniques have been developed to aid with the detection and quantification of *P. cinnamomi* such as PCR and quantitative real time PCR (Eshraghi *et al.*, 2011). PCR offers highly sensitive pathogen detection due to the power of the exponential phase of amplification. Although conventional PCR has aided with the detection and identification of numerous *Phytophthora* species, it has also been shown that when the quantity of target DNA is very small, as with latent infections, the sensitivity of conventional

PCR is inadequate. Most importantly, conventional PCR results are not quantitative. Real time PCR allows fast, reliable and accurate detection and quantification of plant pathogens (Martin *et al.*, 2000). Detection methods using qPCR provide better sensitivity and less variability compared to other non-PCR based techniques (Li *et al.*, 2008). Real time PCR has become a widely used method in plant pathology for accurate detection and quantification of pathogens in infected plants, even at very low infection levels. In real time PCR, DNA is quantified based on the threshold cycle (Ct) value measured at the early exponential stage of the amplification. Sensitivity of real time PCR can be greatly enhanced by implementing a nested approach where 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 a nested PCR two primer pairs are designed based on the sequence of a target gene, where one primer pair is nested within the other. Specificity of real time and real time nested PCR can be assessed by gel electrophoresis, melting curves and sequencing data (Martin *et al.*, 2000).

The degree of pathogen colonization within a plant may sometimes correlate with resistance or susceptibility to a pathogen. Real time PCR is an ideal tool that can be implemented to pick up small changes in host resistance or susceptibility. Qi and Yang (2002) were able to show that resistance of rice cultivars to *Magnoporthe grisea* could be accurately evaluated with real time PCR. By the time lesion development became visible, *M. grisea* was found to be 80 times higher in a susceptible rice cultivar when compared to the resistant plant. Another study on alfalfa plants infected with *Phytophthora medicaginis* (Vandemark & Barker, 2003) indicated significant correlations between the amount of *P. medicaginis* and disease severity. All of these studies support the use of real time PCR to assess the susceptibility / tolerance of host plants and therefore aid in the process of selecting more tolerant varieties. Quantitative measurements of *P. cinnamomi* present within avocado plants can aid in characterizing host resistance or susceptibility towards *P. cinnamomi.* This can also be implemented to study the colonization of the pathogen in different plant tissues.

We developed a nested quantitative PCR method for *in planta* quantification of *P. cinnamomi* in two avocado rootstocks displaying different levels of tolerance against this important soil-borne oomycete. 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 was provided by Westfalia Technological Services (WTS), situated in Tzaneen, Limpopo, South Africa. In addition, 21 *Phytophthora* spp. used for primer specificity assessment were obtained from the culture collection of the Forestry and Agricultural Biotechnology Institute (CMW), University of Pretoria (Table 1). Nine-month-old avocado plants of two commercial rootstocks, R0.09 (highly tolerant) and R0.12 (less tolerant), were also provided by WTS.

Pathogen inoculation and sample collection

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 1 liter, autoclaved at 121°C for 15 min) for five days. Small agar blocks (10 mm 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 1 L dH_2O) was added and incubated for at least three days at room temperature (ca. 25 ºC). Once sufficient mycelial growth was seen, the broth was removed and agar blocks containing mycelia were rinsed three times with sterile H_2O after which 25 ml of Whatman 1 mm-filtered stream water

was added to each plate. Plates were then incubated for 2 – 3 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 ºC for 45 min after which they were removed and left at room temperature for one hour to stimulate zoospore release. The zoospore suspension was removed from the plates, pooled together and used for inoculation.

Inoculation and sample collection

Avocado roots were submerged for an hour in a 5 L container containing zoospore suspension at a concentration of 7.2 x 10^4 ml^{-1} (mock inoculated plants were submerged in sterile water) after which they were transplanted into 1.5 L plastic bags filled with perlite. 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 five 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 ºC. Prior to harvesting, roots were evaluated for root rot symptoms.

DNA extractions

DNA from pure *Phytophthora* cultures was extracted by using PrepMan™ Ultra Reagent (Applied Biosystems, Foster City, California, USA). Mycelia were harvested (ca. 50 mg) and placed in a 1.5 ml eppendorf tube together with 100 µl of Prepman reagent. Tubes were heated for 5 min at 95 ºC, where after the mycelia were homogenized in the tube by using a micro pestle and further incubated for 5 min at 95 ºC, followed by centrifugation for 10 min at 10 000 rpm. Supernatant was collected and diluted five times with sterile water. 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.* (2001).

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 *Actin* gene. The primers Actin-fwd (5'-GTATTCATTCACCACTACTG-3') and Actin-rev (5'- AGTCAAGAGCCACATAAG-3') were designed based on a *Persea americana* Actin sequence available on 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.* (2008) did not yield reliable results in this study. Therefore we selected a new target gene for pathogen quantification. This was the multi copy gene, *Lpv*, which encodes putative storage proteins in zoospores of *P. cinnamomi* (Marshall *et al.*, 2001). 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*. (2003). 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. 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 using the same protocol as for the sample.

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 FastStart *Taq* DNA polymerase (Roche Applied Science, Mannheim, Germany) and 20 to 50 ng of template DNA. Amplification conditions were as follows: 95 ºC for 5 min, followed by 15 cycles at 95 ºC for 30 sec, 55 ºC for 30 sec, and 72 ºC for 30 sec, with a final elongation step at 72 ºC for 10 min. Amplifications were performed in an Eppendorf MasterCycler® gradient (Eppendorf, Hamburg, Germany).

Quantitative real-time PCR

Quantitative real-time PCR was performed using the Bio-rad® CFX 96 instrument. A 20-µl reaction for PCR amplification contained 10 µl Sensimix™ SYBR No-ROX Kit (Bioline Ltd., London, England), 1 µl of each of the forward and reverse primer (10 µM), 2 µl template (2 µl of first PCR product with LPV3 was used as template) 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 sec denaturing at 95 °C, 15 sec annealing at 60 °C and 15 sec 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 sec denaturing at 95 °C, 5 sec annealing at 60 °C and 5 sec primer extension at 72 °C. Control treatments contained water as template. All PCR reactions were performed in triplicate on each of the five biological replicates. Melting curves of real time products were acquired at the end of the PCR run over the range of 65–95°C, increasing the temperature stepwise by

0.5°C every 5 sec 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) (Ginzinger, 2002). Data from the study was analyzed using ANOVA and the software package of JMP'9 (SAS Institute, Inc., Cary, NC) using an LSMeans Differences Student's t-test. In all cases significance was evaluated at $P < 0.05$.

RESULTS

Primer design

Real time primer pairs designed for actin and LPV3N amplified the expected single bands of 77 bp for both products (Table 2). No amplification was observed when actin primers were tested on *P. cinnamomi* DNA or when LPV3N primers were tested with *P. americana* DNA, indicating no cross-specificity. The nested primer set developed by Schena *et al.* (2008) failed to amplify any product and was excluded from further use in this chapter.

PCR efficiencies and linearity

Known concentrations of tenfold serially diluted DNA from *P. cinnamomi* and *P. americana* were used to construct standard curves (Fig. 3). Primer pairs showed high qPCR efficiency rates with high linearity (Fig. 3). 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 as shown in Figure 1. No PCR product was detected when the amount of DNA decreased beyond 20 pg. When nested PCR was used, the detection limit was increased to 20 fg. It is clear that with nested PCR, the sensitivity of detection was significantly enhanced.

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 highly specific for *P. cinnamomi* and only produced a fragment of 70 bp from *P. cinnamomi.* No cross-amplification was achieved 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) indicating the primers were specific for their target sequences (Fig. 4).

In planta monitoring of P. cinnamomi

The growth process of *P. cinnamomi* was assessed for three weeks in inoculated avocado rootstocks by qPCR. Total genomic DNA was extracted from infected root materials which represented both plant and pathogen DNA. The amount of plant DNA was measured by using primers (Actin-fwd and Actin-rev) specific for the avocado *actin* gene. For measuring *P. cinnamomi* DNA, primers (LPV3-fwd, LVP3-rev and LPV3N-fwd and LPV3N-rev) that amplified the *LPV* gene 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 investigated time points, in which the earliest time point was as early as 3 days post infection. 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 (R0.09) and the less tolerant rootstock (R0.12), however, the amount of *P. cinnamomi* DNA was much higher in R0.12 at all time points except day three (Fig. 5). At 3 dpi, there was 3.73 ng of *P. cinnamomi* DNA for every 100 ng of plant DNA in R0.12 and 1.75 ng of *P. cinnamomi* DNA per every 100 ng of plant DNA in R0.09. This relationship reached a maximum amount at 7 dpi, in which there was 15.12 ng of *P. cinnamomi* for every 100 ng of plant DNA in R0.12 and 4.98 ng of *P. cinnamomi* DNA per every 100 ng of plant DNA in R0.09. The ratio decreased significantly at 14 dpi (10.31 ng per 100 ng and 2.34 ng per 100 ng for R0.12 and R0.09 respectively). From 14 dpi to 21 dpi, the ratio of pathogen DNA to plant DNA in root tissue decreased in both rootstocks, however the ratio was significantly higher in R0.12 when compare to R0.09 across this time point (Fig. 5). The significantly higher ratio of *P. cinnamomi* DNA to plant DNA in R0.12 at each time point when compared to R0.09 correlated well to the appearance of the roots, where more lesions and black roots were present in R0.12 than in R0.09 (Fig. 2).

DISCUSSION

This study describes the development and assessment of a nested real time PCR that allowed specific, sensitive and quantitative detection of *P. cinnamomi* within different tissues of the host plant and demonstrated the applicability of the assay to evaluate the infection severity of artificially or naturally infected plant materials. We assessed the disease severity and development of infection in two avocado rootstocks. This is the first report where nested real-time PCR is used to quantify *P. cinnamomi* in avocado. This assay was very sensitive, with detection limits as low as 20 fg of *P. cinnamomi* DNA. This is a significant improvement in sensitivity as other DNA based detection methods were not able to detect such low

concentration (Schena *et al.*, 2008). This supports the fact that nested PCR approach has proven to be very valuable as sensitivity is a key issue in detecting pathogen from soil and plant samples (Judelson & Tooley, 2000).

Previously designed primers (LPV3-fwd and LPV3-rev) for the *Lpv 3* gene by Kong *et al*. (2003) successfully amplified *Lpv 3* 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*, has a very unique sequence for this species. It is a tandem repeat gene that consists of 12 - 18 highly conserved 534 bp repeat units (Marshall *et al.*, 2001). By selecting this gene and placing primers in this repetitive unit, the specificity and sensitivity of the system can be improved. The nested primer pairs designed by Schena *et al. (*2008), which were based on a single copy gene, the ras related protein gene *Ypt1*, did not successfully amplify any product when DNA from infected avocado root tissue was used. However, this could be due to the sensitivity not being adequate to detect low concentration of *P. cinnamomi* in plant material. These primer pairs have only been shown to successfully detect *P. cinnamomi* DNA from pure cultures and not from infected plant tissue (Schena *et al.*, 2008).

For the assay developed in this study, the amount of pathogen DNA is directly normalized with the host plant DNA and therefore provides 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 inhibition is standardized for both plant and pathogen DNA. Moreover, the use of a plant *actin* gene also serves 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. This has been proven in a study

of Diguta *et al*. (2010) with the necrotic fungus *Botrytis cinerea* on grapes where they noted inaccuracy of pathogen quantification when normalizing to host DNA. However, this should not influence the assessment of susceptibility / tolerance of different rootstocks as a less tolerant rootstocks is assumed to have more pathogen 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 (Diguta *et al.*, 2010; Eshraghi *et al.*, 2011).

Maximum amount of *P. cinnamomi* DNA in infected roots was observed at 7 dpi for both the highly tolerant (R0.09) 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 (Fig. 2 and Fig. 5). It is also noteworthy to mention that the maximum amount of *P. cinnamomi* DNA in root tissue of avocado was detected much earlier than the appearance of above-ground symptoms, pointing out the advantage of using this technique in evaluating the tolerance / susceptibility of rootstocks.

Quantitative PCR could be used to reveal the nature of resistance of the plant host to the pathogen (Vandemark & Barker, 2003). In certain cases, low levels of pathogen DNA in resistant plants would indicate a mechanism that result in the inhibition of pathogen multiplication whereas the presence of relatively high amounts of pathogen DNA should indicate a mechanism based on tolerance rather than on true resistance (Schena *et al.*, 2004). In the case of our study there was a significant difference between levels of *P. cinnamomi* in the tolerant and less tolerant rootstock. In this study, the amount of *P. cinnamomi* DNA in avocado roots as determined by real time PCR correlated well with the level of tolerance observed. The amount of *P. cinnamomi DNA* in roots of highly tolerant R0.09 was significantly lower ($P<0.05$) when compared to that of the less tolerant R0.12 at all time points except for 3 days. Pathogen DNA quantified in roots ranged from 3.73 – 15.12

ng per every 100 ng of plant DNA for R0.12 and 0.97 – 4.98 ng per every 100 ng of plant DNA for R0.09, emphasizing the two different levels of tolerance of these rootstocks.

Dan *et al.* (2001) 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 an excellent 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*.

CONCLUSION

This study provides the industry with a valuable tool in the form of an assay which could be utilized for in *planta* monitoring of *P. cinnamomi* in avocado rootstocks. It allows quantification and comparison of the level of infection in rootstocks with varying levels of tolerance, as shown with R0.12 (less tolerant) and R0.09 (highly tolerant). This is the first report to correlate 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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FIGURES AND TABLES

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		+
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	*Cussonia paniculata*	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	_
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	*Vitis vinifera*	South Africa, Western Cape	19411	_
Phytophthora crytogea	Pinus sp	South Africa, Western Cape	19410	_
Phytophthora drechsleri	*Solanum tuberosum*	Argentina, Cordoba	28869	_
Phytophthora drechsleri	*Beta vulgaris*	USA, California	28870	_
Phytophthora eucalypti sp nov	Eucalyptus sp	South Africa, Kwazulu-Natal, Ingwe	22024	_
Phytophthora eucalypti sp nov	*Eucalyptus smithii*	South Africa, Kwazulu-Natal	22029	_

Species	Host	Location	CMW	PCR result Amplified by LPV3N
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	*Medicago sativa*	Canada, Ontario, Dundas County	28865	_
Phytophthora megasperma	*Medicago sativa*	Canada, Ontario, Dundas County	28864	_
Phytophthora multivora	Soil	South Africa, KZN, Umtamvuna Nature Reserve, Port Edward	35209	_
Phytophthora multivora	Soil	South Africa, KZN, Umtamvuna Nature Reserve, Port Edward	35210	_
Phytophthora nicotianae	Citrus sp	South Africa, Limpopo, Tzaneen	19442	_
Phytophthora nicotianae	*Acacia mearnsii*	South Africa, Kwazulu-Natal, Lion River	19443	_
Phytophthora palmivora	*Kentia Palm*	Australia, Queensland, Caboolture	29599	_
Phytophthora palmivora	*Arecastrum romanzoffianum*	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	_
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	_

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

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

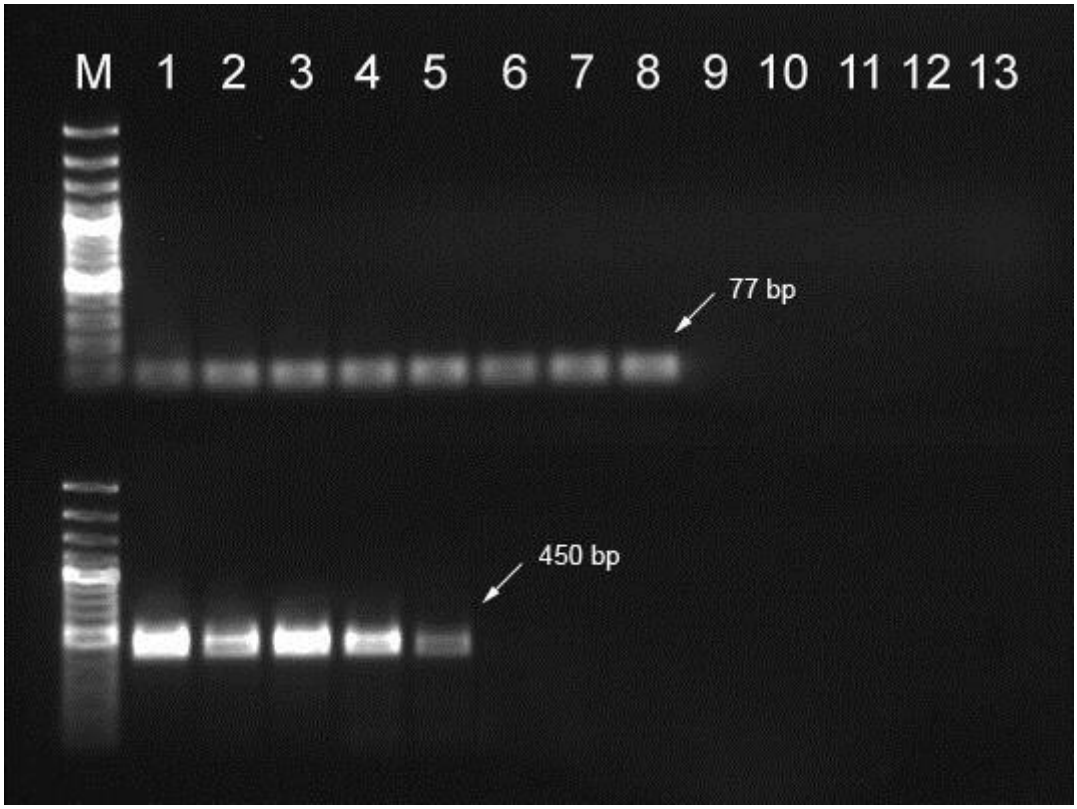

Figure 1. Top - Sensitivity analysis of nested PCR for *Phythophtora cinnamomi*. The first-round PCR was performed using LPV3 as primer pair, and indicated amounts of *P. cinnamomi* DNA as template. The second-round PCR was performed using LPV3N as primer pair and amplified products from first-round 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.

Figure 2. Root symptoms on R0.09 (highly tolerant) and R0.12 (less tolerant) rootstocks after *Phytophthora cinnamomi* infection: A. R0.12 roots at 0 dpi. B. R0.12 roots at 3 dpi. C. R0.12 roots at 7 dpi. D. R0.12 roots at 14 dpi. E. R0.09 roots at 0 dpi. F. R0.09 roots at 3 dpi. G. R0.09 roots at 7 dpi. H . R0.09 roots at 14 dpi.

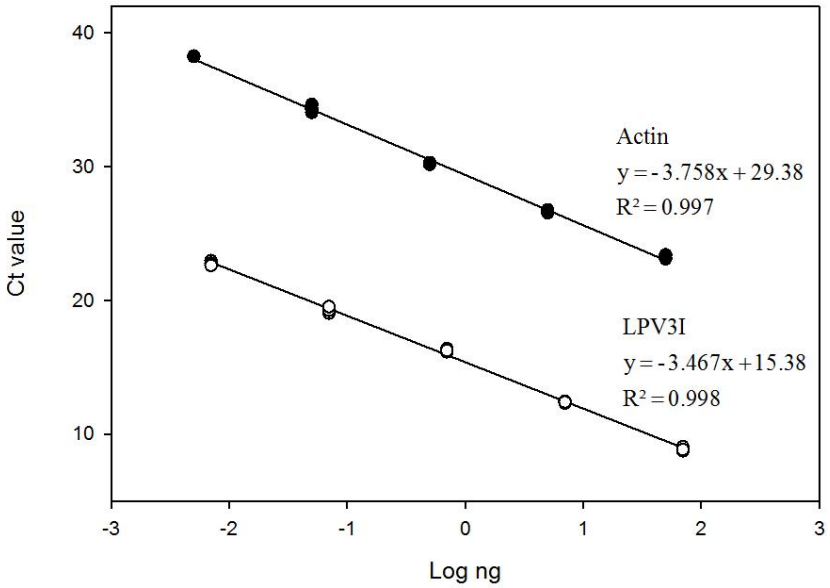

Figure 3. Standard regression curve plots to assess the sensitivity of the qPCR assay. A dilution series of avocado and *Phytophthora cinnamomi* DNA spanning five orders of magnitude (1:0, 1:10, 1:100, 1:1000, 1:1000) amplified with Actin and LPV3N was used to generate standard curves for each separate primer pair.

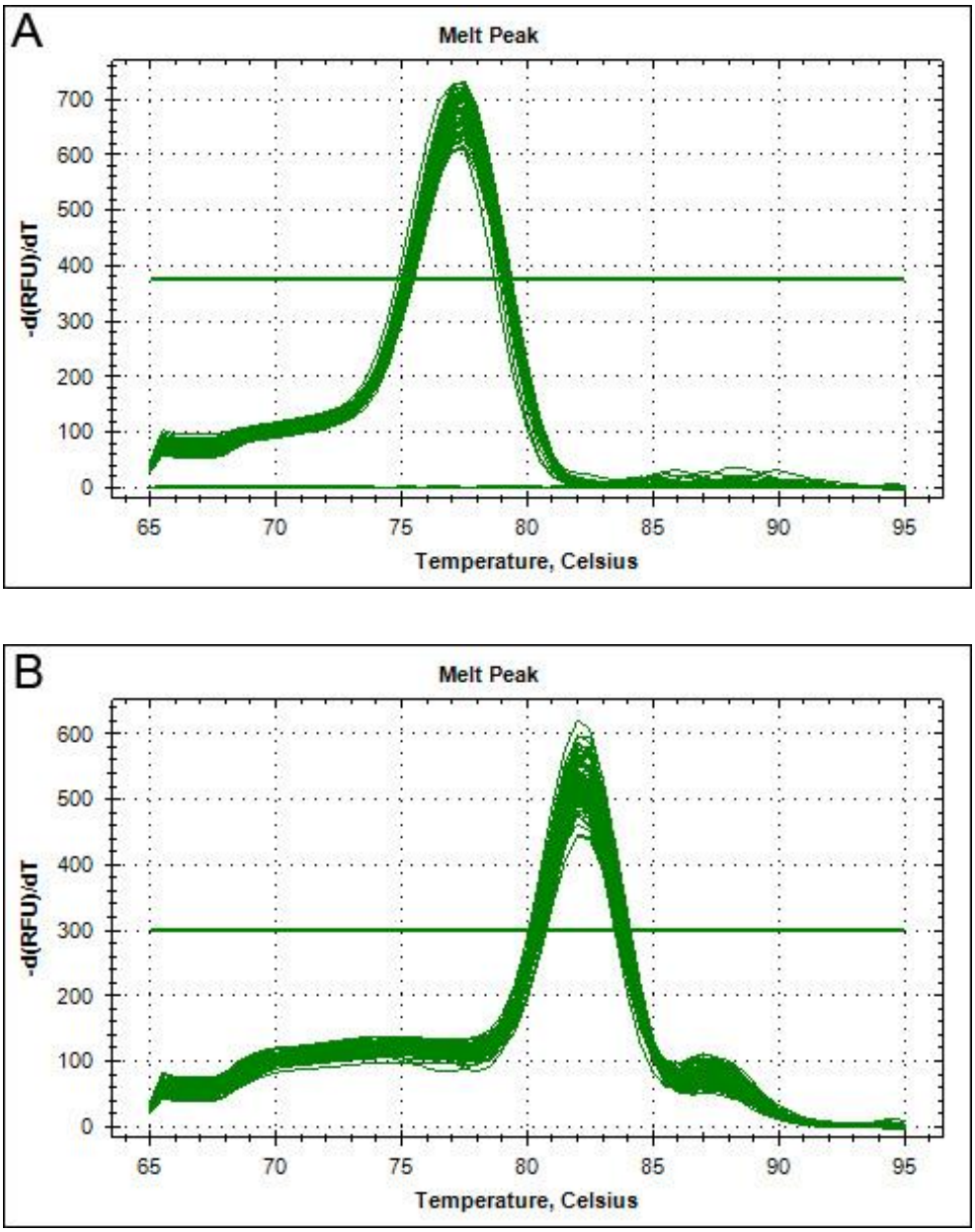

Figure 4. Melting curve analysis for A) Actin and B) LPV3 samples assayed by qPCR. The negative first derivative of the normalized fluorescence was plotted against the temperature to determine the melting temperature (T_m) of the amplicons generated during qPCR analysis.

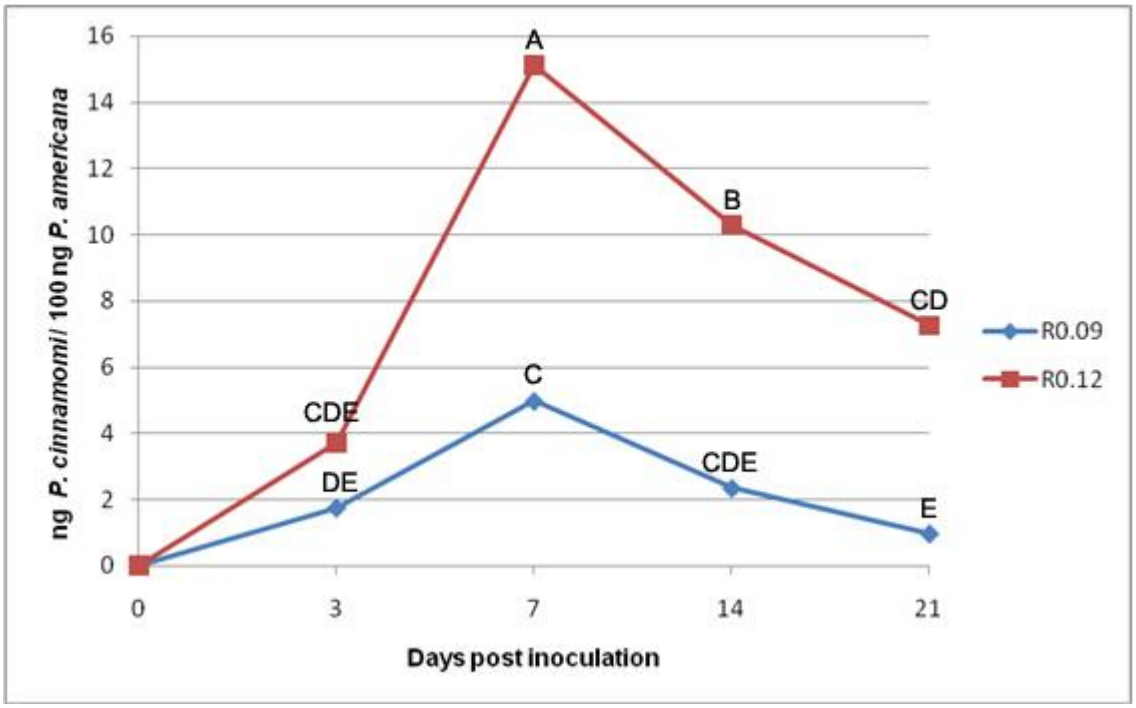

Figure 5. Monitoring of *Phytophthora cinnamomi* growth by qPCR in inoculated avocado root tissues. Pathogen load was quantified from infected root tissues by normalizing the LPV3N values with the corresponding Actin values for each individual sample. Data were analyzed using ANOVA and the software package of JMP 9 using a students t-Test. Bars represented with the same letter are not significantly different at $P>0.05$.

CHAPTER 5

General Discussion

Phytophthora cinnamomi Rands is one of the most well-known oomycetes causing disease in crop plants. It is the causal agent of Phytophthora root rot (PRR) of avocado *(Persea americana* Mill) which results in branch die-back and eventual tree death leading to huge economic losses. Control is achieved through an integrated approach that includes phosphite trunk injections, mulching and the use of tolerant rootstocks. Worldwide crop producers are moving away from chemical control in an attempt to reduce the negative impact of pesticides and insecticides on the environment. Furthermore reports have indicated reduced sensitivity of *P. cinnamomi* to phosphite after prolonged use in avocado orchards (Dobrowolski *et al.*, 2008). Greater emphasis is now placed on alternative options such as the use and development of resistant avocado rootstock varieties to ensure the sustainable production of avocado globally.

Several rootstock varieties have been selected over the years based on their high levels of tolerance against *P. cinnamomi.* These include 'Duke 7', 'Thomas', 'Toro Canyon', 'Martin Grande', 'Spencer' and 'G755' and 'Dusa®'. However, despite the superior performance of these rootstocks under high levels of *Phytopthora* pressure the mechanism of tolerance remains to be elucidated. To date, the specific mechanisms that play a role in defense against *P. cinnamomi* in avocado have not been discovered. Our knowledge is based on the results obtained from the model crop *Arabidopsis* when infected with *P. cinnamomi* (Rookes *et al.*, 2008). Responses such as reactive oxygen species (ROS) induction, hypersensitive response and lignin and callose production were found as well as the involvement of the ethylene and jasmonic pathway in the general defense response. Only three studies have focused on the interaction between avocado and *P. cinnamomi* (García-Pineda *et al.*, 2010; Mahomed & Van den Berg, 2011; Muñiz *et al.*, 2012). García-Pineda *et al.* (2010) found salicylic acid to be a major inhibitor of pathogen colonization on avocado, this is in contrast with ethylene and jasmonic acid as pointed out by Rookes *et al.* (2008). These differences can be explained as *Arabidopsis* is a non-host of *P. cinnamomi.* It is noteworthy to mention that a highly susceptible rootstock was used in the study of Garcia-Pineda *et al.* (2011). For a better understanding of

tolerance in avocado, it is important to use rootstocks with different levels of tolerance against *P. cinnamomi* as was done in our study.

In order to study any host-pathogen interaction it is important to establish a reliable inoculation technique. The two small plant inoculation systems (hydroponics and perlite) confirmed the PRR-tolerance status of avocado rootstocks R0.09, R0.10 and R0.12. R0.09 had the highest level of tolerance against PRR, R0.10 was moderately tolerant and R0.12 was the least tolerant developing black, brittle necrotic lesions on the roots resulting in leaf drop. However, R0.12 was previously selected as having PRR-tolerance but was discarded after failing during field trials. In future studies it would be more valuable to include a completely susceptible rootstock. In addition to conducting pathogenicity assays the hydroponics system also provided root material for down-stream molecular experiments such as transcript profiling (Chap. 3), microscopy (Christie, 2012) and transcriptome sequencing (Mahomed & Van den Berg, 2011). The hydroponics system is however not suitable for prolonged periods as avocado is sensitive to flooding. In cases where late time points are of interest avocado plants should be planted in a growth substrate such as perlite.

This thesis contributed new insight into seven defense associated genes expressed in five avocado rootstocks upon attack by the ubiquitous *P. cinnamomi*. *Phenylalanine ammonia-lyase, lipoxygenase, pathogenesis-related protein* 5, *endochitinase*, *gluthathionine S-transferase, heat shock protein* and *metallothionein* were selected based on their role in plant defense against pathogens and had not previously been studied in the *P. cinnamomi* - avocado interaction using zoospore infection. At the onset of this project only 16558 avocado EST's were available on the NCBI database, with the majority being from fruit and flower material. Defense mechanisms in plants are mediated by a multitude of transcription factors and genes coding for signalling molecules and defense proteins. Differences in gene expression are therefore responsible for phenotypic and genotypic differences; therefore gene expression analysis could help to determine the genes that contribute towards the tolerant phenotype we observe in certain avocado rootstocks. Quantitative gene expression data

indicated that all transcripts investigated were involved in the avocado response after *P. cinnamomi* infection. The data did not allow us to confer an exact role for the transcripts in conferring tolerance against *P. cinnamomi*. This was due to the fact that the different rootstocks often shared similar expression patterns for the genes under investigation. *PR 5* was found to be significantly up-regulated at 48 and 72 hpi in all rootstock varieties infected with *P. cinnamomi*. The continuous up-regulation of *PR 5* across all avocado rootstocks suggests that expression of this gene could be an important early defense strategy against the biotrophic *P. cinnamomi* and that it may have an important role in SAR. The less tolerant R0.12 rootstock showed no significant changes in *endochitinase* expression during the first 24 hours. This delay of 24 hours in R0.12 could be advantageous to *P. cinnamomi* by allowing a longer period for establishment of infection as compared to rootstocks that showed an early significant up-regulation of *endochitinase* expression. Scavengers of reactive oxygen species (ROS) such as *glutathione-S-transferase* and *metallothionein* (Morita *et al.*, 1999) were expressed at constant levels with very few significant changes in expression. Although the involvement of these genes cannot be verified in defense, it is possible that the highly tolerant plants are able to maintain an equilibrium that is sufficient to protect from ROS. The phenylpropanoid pathway also appears to be involved in the defense response against *P. cinnamomi.* A clear trend of down-regulation could be observed for *phenylalanine ammonia-lyase (PAL)* in the susceptible rootstocks and we hypothesizes that this allows the establishment of *P. cinnamomi* in avocado root tissues. This distinct down-regulation was not observed in the more tolerant rootstocks implying that the higher levels of *PAL* may be linked to PRR tolerance.The fact that *lipoxygenase* in R0.12 remained unchanged during the first 24 hours could be an indication that signalling is delayed and thus defense responses are activated too late to restrict the pathogen.

This study has provided some information as to the involvement of known defense genes in avocado but has highlighted the fact that the molecular mechanism underlying PRR-tolerance is regulated by multiple genes. In order to fully explain the role of a specific gene in tolerance

it would be of great value to include a truly susceptible avocado rootstock. Expression data should indicate that some genes are not up-regulated in the susceptible responses or they may even be down-regulated as opposed to the tolerant interaction. Gene expression studies should also be complimented with proteomics to shed light on the proteins involved in disease tolerance. Functional genomics would be invaluable in proving the exact role of a gene but this is hampered by the difficulty to produce transgenic avocados.

Finally the thesis reports on the development of an accurate low cost assay to quantify *P. cinnamomi* within avocado rootstocks. A nested primer set was designed for the *Lpv* gene coding for a storage protein specific to *P. cinnamomi*. The *Lpv* gene-coding region contains 12–18 highly conserved 534-bp repeats, flanked by unique sequences. This region is unique for *P. cinnamomi* and therefore has great potential for developing a specific and sensitive DNA sequence based detection protocol. Primers developed by Kong *et al.* (2003) for *Lpv* were designed to detect *P. cinnamomi* within artificially infested soil and irrigation water from nurseries. However, the assay was not developed in a quantitative manner and also was not sensitive enough to detect *P. cinnamomi* at very low levels within plant tissue. Another available method for detection of *P. cinnamomi* was also developed using a nested PCR approach, but this did not produce reliable results within our study (Schena *et al.*, 2008). Therefore a reliable and sensitive method to detect *P. cinnamomi* in plant tissue is needed and was successfully developed in this study. The quantification of *P. cinnamomi* in R0.09 (highly tolerant) and R0.12 (least tolerant) indicated that tolerance was correlated with the amount of pathogen present within the root tissue, with the more tolerant rootstocks having statistically less pathogen present in the roots. The assay also allowed the detection of *P. cinnamomi*, even before the onset of characteristic root symptoms. This molecular tool is of significance as it has the potential to be used in rootstock selection trials to identify avocado germplasm that show tolerance to PRR. Conventional selection trials are time consuming and are based on visual assessments only. By assessing the amount of pathogen *in planta* test seedlings can be

selected based on quantitative molecular data as opposed to only phenotypic disease symptoms.

This thesis has contributed both basic and applied research results that could be of value to avocado researchers and the industry. The small plant inoculation systems are reliable, providing consistent disease severity data as well as providing root material for different research purposes. The gene expression data provided some of the first insights regarding the role of genes involved in defense and could aid in the identification of key defense pathways which could direct research. Finally, the nested quantitative PCR is a novel assay for the detection of *P. cinnamomi* in avocado roots that can also be correlated with the level of PRR resistance in avocado rootstocks.

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