Characterization of *Phytophthora cinnamomi* from avocado

By

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Declaration

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

Elrea Appelgryn

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Preface

Phytophthora Root Rot (PRR), caused by the hemibiotrophic soil-borne oomycete *Phytophthora cinnamomi* Rands is the most destructive disease of *Persea americana* Mill. (avocado) in South Africa. Typical symptoms of affected trees in an orchard include root rot, stem cankers, branch dieback and a general decline in tree health, resulting in a failure of trees to carry fruit. An integrated approach to control includes PRR-tolerant avocado rootstocks, good management practices and chemical control through the use of phosphonates, which limits losses caused by this devastating pathogen. However, the possible evolution of resistance to chemical control agents currently used, and the recent focus on organic farming practices highlights the importance of selecting avocado rootstocks that are fully resistant to *P. cinnamomi* infection for sustainable avocado production in the future.

The mechanism underlying PRR tolerance in avocado rootstocks is unknown, and thus rootstocks cannot be selected using specific markers associated with tolerance. Currently, potential new tolerant rootstocks are identified through selection of seedlings exhibiting tolerance to artificial *P. cinnamomi* infection in mist bed-and field trials. This is a time-consuming process, which can take up to 25 years. Diversity in pathogen populations, as well as specific pathogen-host interactions determines the durability of resistance in pathosystems. Understanding the infection process of the pathogen and the interaction between *P. cinnamomi* and its host, avocado, at both a molecular and physiological level, will aid investigations into possible mechanisms of tolerance in avocado rootstocks. The amount of diversity present in *P. cinnamomi* isolates will emphasize the importance that the selection of isolates can play during the identification of promising new rootstocks.

The first aim of this study was to establish a transformation system for *P. cinnamomi* using the Green Fluorescent Protein (GFP) marker gene. A fluorescently tagged strain of *P. cinnamomi* is useful for studying the *P. cinnamomi-P. americana* interaction, and the establishment of such a system will also be an invaluable tool in future research and projects, such as the functional characterization of pathogen avirulence factors. After failing to transform a *P. cinnamomi* isolate of US origin during a research visit to the University of California, Riverside (USA), putative transformants were obtained for the South African *P. cinnamomi* isolate, TBB5T7S3, an isolate recently used for transcriptome sequencing of *P. cinnamomi* by the Fruit Tree Biotechnology Programme. However, transformation success may be isolate-specific, which led to the re-evaluation of the amount of phenotypic variation

among *P. cinnamomi* isolates, with a focus on *P. cinnamomi* isolates from avocado orchards.

Chapter 1 entitled "*Phytophthora cinnamomi* Rands, the most destructive pathogen of avocado in South Africa" gives an overview of *P. cinnamomi*, its host, avocado and PRR. Background is given on the host range, disease symptoms, reproduction, infection, control and diversity of *P. cinnamomi*. Four different methods for transformation of *P. cinnamomi*, optimization of protoplasting and transformation, as well as applications of fluorescently tagged pathogens are discussed.

Chapter 2 reports on attempts to optimize protoplasting and PEG and $CaCl_2$ mediated protoplast transformation of *P. cinnamomi*. Mycelia and young germinated spores were compared as starting material, and previously published protocols for protoplasting and transformation were evaluated. Finally, recommendations for alternatives to protoplast transformation are given.

In Chapter 3 the amount of phenotypic variation in *P. cinnamomi* isolates was investigated. Differences in *in vitro* growth rate, sporulation and pathogenicity to avocado were evaluated in 12 *P. cinnamomi* isolates obtained from avocado orchards in the Limpopo, Mpumalanga and KwaZulu-Natal provinces in South Africa, as well as orchards in California, USA. Correlations between the results of different experiments were also investigated.

An overview of the results and findings of this dissertation is discussed in Chapter 4. Final comments on the importance of characterizing *P. cinnamomi* isolates in terms of variation in pathogenicity and amenability to transformation are given, and recommendations for future research is discussed, and concludes with a summary.

List of abbreviations and symbols

°C	Degree Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
%	Percentage
ABC	ATP Binding Cassette
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
BABA	β-aminobutyric acid
bp	Base pair
BS	Bootstrap
CaCl2	Calcium chloride
CaCO3	Calcium carbonate
CMA	Cornmeal agar
CaMV	Cauliflower Mosaic Virus
CBEL	Cellulose Binding Elicitor and Lectin-like
CLSM	Confocal Laser Scanning Microscopy
cm	Centimetre
CRN	Crinkling and Necrosis inducing
CTAB	Cetyltrimethylammoniumbromide
CWDE	Cell Wall Degrading Enzyme
dH2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethyelenediaminetetra-acetic Acid
FPB	Fry Protoplasting Buffer
g	Gram

GFP	Green Fluorescent Protein
GC content	Guanosine Cytosine content
GUS	β-glucorinidase
ha	Hectare
hr	Hour
hpt	Hygromycin phosphotransferse
kb	Kilo bases
KCl	Potassium chloride
l	Litre
LB	Left Border
LiCl	Lithium Chloride
m	meter
M	Molar
MAPK	Mitogen-activated protein kinase
MEA	Malt Extract Agar
MES	2-(N-morpholino)ethanesulfonic acid
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
mM	Milimolar
MP	Maximum Parsimony
MT	Mannitol Tris
NaCl	Sodium chloride
NARPH	Nystatin;Ampicillin;Rifampicin;Pentachloronitrobenzene
NCBI	National Center for Biotechnology Information
ng	Nanogram
NPP1	Necrosis inducing Phytophthora Protein 1
NLP	Nep 1-Like Protein
npt	Neomycin phosophotransferse

PDA	Potato Dextrose Agar
PCR	Polymerase Chain Reaction
PEG	Polyethyeleneglycol
PR	Pathogenesis Related
PRR	Phytophthora root rot
qPCR	Quantitative PCR
R-genes	Resistance genes
RAPD	Random Amplified Polymorphic DNA
RB	Right Border
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	Revolutions Per Minute
RT-qPCR	Reverse Transcriptase quantitative PCR
RXLR	Arginine-any amino acid-Leucine-Arginine
sec	Second
SSR	Simple Sequence Repeat
spp.	Species
TBR	Tree Bisection Reconnection
T-DNA	Transfer DNA
V	Voltage
WTS	Westfalia Technological Services
YM broth	Yeast Malt broth

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Chapter 1

Phytophthora cinnamomi Rands, the most destructive pathogen of avocado in South Africa

Introduction

Species in the genus *Phytophthora* are widely recognized as some of the most devastating plant pathogens, and are responsible for major losses in crops such as potato, tomato, soybean, pepper and alfalfa, and fruit-and forestry trees. The Irish potato famine of 1845 caused by *Phytophthora infestans* (Mont.) de Bary is a historic example of the devastation this group of pathogens can cause (DE BARY 1876). More than 100 species of *Phytophthora* have been identified and described to date, and species of this genus are divided into ten clades (BLAIR *et al.* 2008; KROON *et al.* 2011; MARTIN *et al.* 2014). Research on this group of pathogens is focussed on the identification of host factors interacting with effectors, the impact of each effector on pathogenic fitness and their sub-cellular localization in order to identify proposed roles during infection (HUITEMA *et al.* 2011). This could lead to the application of knowledge on pathogenicity factors to develop novel control strategies.

Phytophthora cinnamomi Rands is a member of clade seven in the *Phytophthora* genus (BLAIR *et al.* 2008; KROON *et al.* 2011; MARTIN *et al.* 2014), and was identified in 1922 following isolation from cinnamon trees in Sumatra (RANDS 1922). Members of clade seven are all non to semipapillate, and can be either homo or heterothallic (KROON *et al.* 2011). *Phytophthora cinnamomi* is a hemi-biotrophic, heterothallic pathogen capable of infecting in excess of 3000 plant species (HARDHAM 2005). These include forestry trees such as *Eucalyptus* and *Pinus* spp., fruit trees such as avocado and peach, and also macadamia and chestnut (HARDHAM 2005). *Phytophthora cinnamomi* is a threat to natural ecosystems and biodiversity, and has had a devastating effect on the indigenous jarrah (*Eucalyptus marginata*) forests in western Australia (PODGER *et al.* 1965). Disease symptoms of *P. cinnamomi* infection include root rot, stem cankers and shoot die-back (HARDHAM 2005). Papua New Guinea has been proposed to be the centre of origin (HARDHAM 2005; ZENTMYER 1988), but today *P. cinnamomi* is present around the globe. The pathogen has been reported in Australia, Mexico, South Africa, Europe and the USA (CAHILL *et al.* 2008). The A2 mating type is more prevalent (ZENTMYER 1988), and is commonly associated with the infestation of crops and forests in South Africa (VON BROEMBSEN 1984).

Phytophthora cinnamomi is an economically important pathogen in South Africa, as it is the causal agent of Phytophthora root rot (PRR) in *Persea americana* Mill. (avocado), and a major limiting factor of both the local and international avocado industry. Avocado rootstocks tolerant to *P. cinnamomi* infection, together with chemical control through the use of phosphite and good farming practices, are utilized to minimize the effect of this pathogen.

However, under environmental stress conditions such as flooding, tolerant rootstocks are not able to completely withstand infection (REEKSTING *et al.* 2014).

Despite the availability of avocado rootstocks tolerant to PRR, the mechanism underlying tolerance or susceptibility in rootstocks is unknown. This complicates avocado rootstock breeding and selection, as they cannot be bred or screened for specific characteristics or markers associated with resistance. Currently, due to the unavailability of specific markers associated with tolerance or resistance, rootstocks that show promise following evaluation for tolerance in mist beds infected with *P. cinnamomi* are subjected to long term field trials, and the whole process of selecting new rootstocks can take up to 25 years. The evolution of resistance to chemical control agents such as phosphite in *P. cinnamomi* is also a threat (DOBROWOLSKI *et al.* 2008), and thus avocado rootstocks that are highly tolerant/resistant to *P. cinnamomi* infection are required for sustainable avocado production in the future. An improved understanding of the interaction between *P. cinnamomi* and its host, avocado, will aid in the identification of resistance mechanisms active in tolerant rootstocks, allowing breeders to select tolerant plants much earlier, greatly decreasing the amount of time required to produce new rootstocks.

The arrival of the genomics era has led to the identification of hundreds of pathogenicity factors in *Phytophthora* spp., contributing to research aimed at understanding the complex interactions between plant defence responses and pathogen molecules. The establishment of transformation systems for many *Phytophthora* spp. has allowed for functional evaluation of pathogenicity factors, but also has other useful applications. Fluorescently-tagged plant pathogens have proved to be useful tools in studies aimed at unravelling plant-pathogen interactions. Applications of fluorescently tagged pathogens include quantitative *in planta* detection of pathogens (SI-AMMOUR *et al.* 2003), the ability to detect pathogens in parts of the plant where their abundance is low, pathogen detection before the onset of visible disease symptoms (LE BERRE *et al.* 2008a), the study of plant-pathogen interactions (BOTTIN *et al.* 1999; PLIEGO *et al.* 2009; RIEDEL *et al.* 2009), tracking of genetically modified strains (BOTTIN *et al.* 1999) and *in planta* evaluation of possible biocontrol agents (LU *et al.* 2004). Fluorescently tagged strains of *P. infestans* have also been used to study the splash dispersal of spores (SAINT-JEAN *et al.* 2005). A stable Green Fluorescent Protein (GFP)-tagged strain of *P. cinnamomi,* together with a successful transformation protocol will be an invaluable tool for future research and projects.

The amount of variation among *P. cinnamomi* isolates with regards to pathogenicity and genotypic diversity has important implications for the South African avocado industry.

Variability in *P. cinnamomi* pathogenicity and specific host-isolate interactions can influence the durability of resistance (ROBIN and DESPREZ-LOUSTAU 1998), thus studies aimed at evaluating the variability among different isolates of *P. cinnamomi* are required. Previous studies on the genotypic and phenotypic variation among South African *P. cinnamomi* isolates were conducted in the 1990s, and focussed on isolates collected from forestry trees and natural vegetation (LINDE *et al.* 1997; LINDE *et al.* 1999a; LINDE *et al.* 1999b). Re-evaluation of the diversity present in South African *P. cinnamomi* isolates is required, and a focus on isolates from avocado orchards can have a significant impact on breeding and screening programmes aimed at producing tolerant or resistant rootstocks.

This review will discuss components of the biology of *P. cinnamomi* that make it such a successful pathogen, its host avocado, and factors that need to be considered when implementing transformation protocols in oomycetes.

Oomycetes

Oomycetes and fungi are eukaryotic plant pathogens responsible for significant losses in agriculture and horticulture, but also lead to damage in natural ecosystems and to biodiversity through the death of native vegetation (HARDHAM 2005). For many years, similarities such as filamentous growth during the vegetative stage and reproduction through sexual and asexual spores resulted in the classification of oomycetes in the kingdom Fungi (LATIJNHOUWERS *et al.* 2003). However, in later years differences in phenotypic characters and analysis and comparison of conserved gene regions, such as the small ribosomal subunit, actin and tubulin, separated fungi and oomycetes into distinct phylogenetic groups **(Figure 1)** (FRY and GRÜNWALD. 2010; LATIJNHOUWERS *et al.* 2003).

Differences in physiology, biochemistry and genetics between fungi and oomycetes are attributed to the fact that fungi and animals (Metazoa) share a common ancestor, while heterokaryont golden-brown algae is the closest relative of the oomycetes (BALDAUF *et al.* 2000; LATIJNHOUWERS *et al.* 2003). Oomycetes are subsequently classified in the kingdom Stramenopiles (HARDHAM 2005; WALKER and VAN WEST 2007), which also includes some of the algae and other fungal-like organisms. Water moulds and downy mildews are further classified in the division Oomycota, and in the class Oomycetes (WALKER and VAN WEST 2007). Members of the Oomycetes all produce biflagellate zoospores, have tubular mitochondrial cristae, rely on the diaminopimelic pathway for lysine biosynthesis, are diploid in the vegetative state and have cell walls of cellulose (β-1,4-glucans), all factors that

distinguish them from the fungi (LATIJNHOUWERS *et al.* 2003; WALKER and VAN WEST 2007). More than 500 oomycete species have been described to date, which includes obligate or facultative plant, animal, fungal, protist and Stramenopile pathogens (JUDELSON 1996; THINES and KAMOUN 2010; WALKER and VAN WEST 2007).

Genus *Phytophthora*

Phytophthora infestans (Mont.) de Bary was the first oomycete to be identified, and was described in 1876 (DE BARY 1876), and until recently, the description of more than 127 other species of *Phytophthora* have followed (MARTIN *et al.* 2014). In 2013 alone, five new *Phytophthora* spp. (*Phytophthora pluvialis* Reeser, Sutton and E. Hansen (REESER *et al.* 2013*)*, *Phytophthora mississippiae* X. Yang, W. E. Copes, and C. X. Hong (YANG *et al. 2013)*, *Phytophthora cichorii* Bertier, H. Brouwer, De Cock & D.E.L. Cooke, *Phytophthora dauci* Bertier, H. Brouwer & De Cock, and *Phytophthora lactuceae* Bertier, H. Brouwer & De Cock (BERTIER *et al.* 2013) and two hybrid spp. were described. Extensive surveys for new species in natural and agricultural environments, coupled with modern DNA identification methods has led to a rapid increase in the number of newly identified *Phytophthora* spp. in the last 15 years (KROON *et al.* 2011; MARTIN *et al.* 2014), and this number is set to increase further.

The focus on *Phytophthora* research has led to the development of useful tools, such as developmental stage-specific EST data, bacterial artificial chromosome (BAC) libraries and genetic linkage maps for some species of the genus (*P. infestans* and *Phytophthora sojae* Kaufmann & Gerdemann) (VAN DER LEE *et al.* 1997; WHISSON *et al.* 1995). A large number of *Phytophthora* spp. has also been transformed with GFP. These include *Phytophthora palmivora* E.J. Butler (VAN WEST *et al.* 1999b), *P. sojae* (CHEN *et al.* 2009), *Phytophthora parasitica* Dastur (*Phytophthora parasitica* Dastur*, Phytophthora parasitica* Dastur var *nicotianae* Breda de Haan and *P. nicotianae* Breda de Haan var.*Nicotianae* Waterhouse all refer to the same species) (LE BERRE *et al.* 2008a), *P. infestans* (AH-FONG and JUDELSON 2011; SI-AMMOUR *et al.* 2003), *Phytophthora brassicae* De Cock & Man in´t Veld (SI-AMMOUR *et al.* 2003) and *Phytophthora ramorum* Werres, De Cock & Man in´t Veld (RIEDEL *et al.* 2009). GFP-tagged pathogens facilitate an improved understanding of the infection and colonization strategies of these pathogens *in planta*.

The rise of the genomics era has affected and greatly changed the research focus of studies undertaken within oomycetes, with a major focus on the identification of genes associated

with pathogenicity. The availability of genome and transcriptome data for *Phytophthora* spp. such as *P. infestans* (HAAS *et al.* 2009; KAMOUN *et al.* 1999; RANDALL *et al.* 2005)*, P. sojae* (QUTOB *et al.* 2000; TORTO-ALALIBO *et al.* 2007; TYLER *et al.* 2006)*, P. ramorum* (TYLER *et al.* 2006) and *Phytophthora capsici* Leonian (CHEN *et al.* 2013; LAMOUR *et al.* 2012) has led to the identification of hundreds of genes associated with pathogenicity. The ability to transform *Phytophthora* spp. has also allowed for functional characterization of gene sequences, and the results of each new study contributes to understanding the complex interaction between species of *Phytophthora* and their hosts.

Phytophthora species differ in host range, lifestyle and mode of infection (LE BERRE *et al.* 2008b). For example, *P. infestans* has a relatively small host range, is dispersed through air and colonizes leaf material, while *P. parasitica*, a soil-borne root and stem pathogen, attacks up to 60 different plant families (LE BERRE *et al.* 2008a). The more than 100 species in this genus are divided into ten clades **(Figure 2)**, and despite the diversity present in the group, the common thread within this genus is that all members are devastating plant pathogens causing severe damage to crops and native vegetation alike.

Phytophthora cinnamomi

Host range and disease symptoms

Phytophthora cinnamomi is found in soil and dead plant material, and has a wide host range, as more than 3000 plant species are known to be affected (HARDHAM 2005). *Phytophthora cinnamomi* causes root rot in trees such as *Eucalyptus* (PODGER et al. 1965), *Pinus* (BUTCHER et al. 1984), *Persea americana* (avocado) (WAGER 1942), *Macadamia integrifolia* (macadamia) (ZENTMYER and STOREY 1961), *Castanea dentata* (chestnut) (VANNINI and VETTRAINO 2001) and *Prunus persica* (peach) (MIRCETICH and KEIL 1970) and also in *Vitis vinefera* (grapevines), the Proteaceae and other endemic fynbos of South Africa (HARDHAM 2005). The roots of especially older host trees become black and brittle, and affected trees in an orchard are easily distinguished by their sparse foliage and their failure to carry fruit. Inefficient transport of water and nutrients from the diseased roots to the shoots can also lead to the dieback of shoots and stem cankers **(Figure 3)** (HARDHAM 2005).

Sexual and asexual reproduction

Species of *Phytophthora* can either be homothallic or heterothallic (HARDHAM *et al.* 1991). Homothallic species are able to produce sexual oospores by selfing, as isolates of opposite mating types are not required for sexual reproduction. *Phytophthora cinnamomi* is heterothallic, with individuals of two different or opposite mating types, A1 and A2, required for sexual reproduction (HARDHAM 2005). Sexual reproduction gives rise to thick walled oospores through gametangial copulation, and involves the injection of non-swimming sperm produced in the antheridium into the oogonium containing the egg cells **(Figure 4)** (HARDHAM 2005; VAN WEST *et al.* 2003). The gametes are the only stage of the life cycle of *P. cinnamomi* that is haploid. Oospores are resistant to extreme environmental conditions and can survive in the soil for prolonged periods of time, and serve as a long term source of inoculum (DUNCAN 1980). When environmental conditions become favourable once again, oospores can germinate to form germtubes, which give rise to sporangia, reinstating the asexual life cycle (VAN WEST *et al.* 2003).

Under conditions favourable for growth the asexual cycle for reproduction dominates (HARDHAM 2005). During asexual reproduction, hyphae or chlamydospores give rise to multinucleate sporangia. Sporangia form at the tips of hyphae through the flow of cytoplasm into the hyphal apex, and are sealed off at maturity by the deposition of a basal septum (HARDHAM 2005; HARDHAM 2007). Sporangia can either germinate directly by producing germtubes, or indirectly by differentiating into zoospores (HARDHAM 2007; VAN WEST *et al.* 2003). Sporangia persist until a signal initiates the cytoplasmic cleavage of the sporangium, which results in the release of 20-30 zoospores via the sporangial apex (HARDHAM 2005; HARDHAM 2007; WALKER and VAN WEST 2007). A high level of free moisture in the soil or chilling leads to the release of the zoospores from the sporangia into the soil (GOODWIN 1997).

Population structure

Despite the large losses in agriculture caused by *Phytophthora* each year, little was known about the population genetic structure of these species (GOODWIN 1997). In the past, the inability to perform genetic crosses due to homothallism, the failure of oospores to germinate and limited access to both mating types and suitable markers made population genetics studies on *Phytophthora* spp. very difficult (SHAW 1991). In the last number of years however, the number of population studies on *Phytophthora* spp. has increased (COOKE *et*

al. 2005; DURAN *et al.* 2010; IVORS *et al.* 2004). A number of population studies, using markers such as isozymes, restriction fragment length polymorphisms (RFLPs) and simple sequence repeats (SSRs), have been completed on *P. cinnamomi* isolates from mainly South Africa and Australia.

Isozyme analysis of South African isolates indicated low levels of genetic and genotypic diversity, and no evidence for sexual recombination was found (LINDE *et al.* 1997). However, the limited number of isozyme loci and alleles at those loci restricted population genetic studies (MICHELMORE and HULBERT 1987). This limitation was overcome by using microsatellites, random amplified polymorphic DNA (RAPDs) and RFLPs to better investigate the relationships between isolates (DOBROWOLSKI *et al.* 2003; LINDE *et al.* 1999a). A follow-up study using RAPDs and RFLPs as markers by Linde and co-workers on the population genetic structure of *P. cinnamomi* in South Africa and Australia confirmed the results obtained by the earlier study using isozymes as markers (LINDE *et al.* 1999a). The genetic distance between the South African and Australian populations was very low and the two populations shared a large number of alleles. Both studies using different markers were indicative of a lack of sexual reproduction, and the authors postulated that both the South African and Australian populations were introduced (LINDE *et al.* 1999a).

In Australia, only three SSR multilocus genotypes were identified (one of the A1 mating type, and two of the A2 mating type), and no evidence of sexual reproduction among the isolates were found (DOBROWOLSKI *et al.* 2003; OLD *et al.* 1984). The exact same three multilocus genotypes were also reported in other parts of the world together with three additional genotypes of the A1 mating type. Comparisons between the Australian and South African isolates revealed genotypes common to both (LINDE *et al.* 1999a). These observations lead researchers to hypothesize that *P. cinnamomi* dispersed around the world through clonal spread, as *P. cinnamomi* isolates from around the world all exhibit low levels of genetic and genotypic variation (DOBROWOLSKI *et al.* 2003; HÜBERLI *et al.* 2001; LINDE *et al.* 1997; LINDE *et al.* 1999a).

Isolates from Papua New Guinea show uncommonly high levels of genetic diversity (OLD *et al.* 1984). Apart from the multilocus genotypes found around the world, an additional seven multilocus genotypes of the A1 mating type were identified. Researchers have subsequently postulated that Papua New Guinea is the possible centre of origin of *P. cinnamomi* (OLD *et al.* 1984). Knowing where the centre of origin is would enable scientists to select for disease resistance in plants that area, and could improve quarantine procedures to limit the spread of genotypes (LINDE *et al.* 1999a).

Hemibiotrophic infection strategy

Many *Phytophthora* spp. are hemibiotrophs, as they typically follow a biotrophic life style after infection, but gradually switch to a necrotrophic phase later in the infection cycle. In biotrophic interactions, host cells are not killed by the pathogen, while they are destroyed by necrotrophic pathogens, after which they feed on the host as saprophytes. During the biotrophic stage of infection, pathogens commonly try to evade plant defence responses by either actively suppressing defence, or by avoiding detection to prevent the activation of plant defences (MÜNCH *et al.* 2008). During the subsequent necrotrophic phase of infection, the pathogen kills host cells with the host no longer capable of attempting a defence response (MÜNCH *et al.* 2008).

According to Mendgen and Hahn (2002), biotrophy relies on "highly developed infection structures, limited secretory activity (especially of lytic enzymes), carbohydrate-rich and protein-containing interfacial layers that separate fungal and plant plasma membranes, long-term suppression of host defence and haustoria for nutrient absorption and metabolism" (MENDGEN and HAHN 2002). Biotrophs are obligate (SCOTT 1972), minimize host damage, and are specific with limited host ranges (LEWIS 1973). Plants often respond to attack by biotrophic pathogens through salicylic acid-dependent defence pathways, and gene-for-gene interactions usually underlie resistance (BARNA *et al.* 2012; HAMMOND-KOSACK and PARKER 2003; OLIVER and IPCHO 2004).

In comparison, necrotrophs are non-obligate pathogens with wide host ranges, which secrete both cell wall degrading enzymes, as well as other toxins (BARNA *et al.* 2012; HAMMOND-KOSACK and PARKER 2003; LEWIS 1973). Jasmonic acid and ethylene-dependent defence pathways are activated upon challenge with necrotrophic pathogens, and quantitative resistance genes underlie plant resistance to necrotrophs (HAMMOND-KOSACK and PARKER 2003). It is widely accepted that salicylic acid- and jasmonic acid-dependent defence pathways act antagonistically, implicating that defence against biotrophs and necrotrophs will also be antagonistic (OLIVER and IPCHO 2004). Salicylic acid-dependent defence responses is thus proposed to be active in the early stages of defence, followed by the activation of jasmonic acid-associated defence responses later in the infection (BARNA *et al.* 2012).

Asymptomatic infection of native Australian plants by *P. cinnamomi* prompted Crone *et al.* (2012) to suggest that *P. cinnamomi* infection does not always include a necrotrophic phase (CRONE *et al.* 2012). Shearer and Crane (2012) proposed that *P. cinnamomi* might alternate between biotrophic and necrotrophic trophic lifestyles, depending on environmental

conditions (SHEARER and CRANE 2012). Classification of the trophic lifestyle of *P. cinnamomi* may thus require re-evaluation in specific hosts, but the infection of *P. americana* by *P. cinnamomi* corresponds to hemibiotrophy (MAHOMED and VAN DEN BERG 2011), and thus *P. cinnamomi* will be considered a hemi-biotroph in this study (CAHILL *et al.* 2008; DAVISON *et al.* 1994).

Infection process

Oomycetes and fungi have to overcome the same plant defences, whether structural or chemical, to enable infection (HARDHAM 2007). The main difference between the infection strategies of fungi and oomycetes is that the latter have motile zoospores which can actively target suitable infection sites (HARDHAM 2007; ZENTMEYER 1961). Uninucleate and wall-less zoospores are the principal infective agent of *P. cinnamomi* (HARDHAM 2005; LATIJNHOUWERS *et al.* 2003).

Motile zoospores are biflagellate, with two morphologically distinct flagella (HARDHAM 2005; WALKER and VAN WEST 2007). The posterior flagella, which is of the whiplash type is longer than the anterior flagella, and is responsible for steering the zoospore. The anterior flagella, a tinsel type, pulls the zoospore through water (HARDHAM 2007; WALKER and VAN WEST 2007). Zoospores are able to move at speeds of 200 µm/s (HARDHAM 2005), and can cover distances of a few centimetres in soil, ensuring rapid dispersal between hosts.

Zoospores have two major responsibilities in the infection process: first to mediate transmission between hosts, and secondly, to select a suitable site for infection (WALKER and VAN WEST 2007). Motile zoospores detect suitable infection sites on host and non-host plants through three mechanisms: chemotaxis, autotaxis and electrotaxis (HARDHAM 2007; WALKER and VAN WEST 2007). During chemotaxis, zoospores are attracted towards hosts in response to chemicals such as sugars, amino acids, alcohols and phenolic compounds released by the host (HARDHAM *et al.* 1991; WALKER and VAN WEST 2007; ZENTMEYER 1961). These molecules are commonly found in the rhizosphere, thus zoospores are often attracted to both susceptible and resistant hosts. This implies that chemotaxis is not a resistance mechanism, and that the mechanisms underlying resistance are active at later stages in the infection process. Chemotaxis does, however, target zoospores to specific regions on the root. Zoospores typically attach to the elongation zone behind the root cap, possibly because the plant cell walls in this region are more easily penetrated than cell walls in more mature regions of the plant (HARDHAM 2001a).

Autotaxis involves the aggregation of zoospores in groups in response to chemicals (WALKER and VAN WEST 2007). It has been proposed that high nutrient or calcium levels in soil might stimulate zoospores to swim in groups ranging from hundreds to thousands. The aggregation of zoospores is species-specific, as zoospores only aggregate with other spores of the same species. Through electrotaxis, ionic currents circulating in plants result in the formation of electrical fields, which serve to attract zoospores to specific plant regions, such as regions between epidermal cells (WALKER and VAN WEST 2007). Once a suitable host has been located, zoospores orientate their ventral surface toward the plant surface, the flagella are shed or retracted (WALKER and VAN WEST 2007), and zoospores form germinating cysts (HARDHAM 2007; LATIJNHOUWERS *et al.* 2003).

Upon encystment, *Phytophthora* spp. release an adhesive substance stored inside specialized vesicles in the zoospore, which aid in zoospore attachment to the plant surface, and this is accompanied by deposition of a cell wall for protection (HARDHAM 2005; HARDHAM and SUZAKI 1986; IRVING and GRANT 1984; LATIJNHOUWERS *et al.* 2003; WALKER and VAN WEST 2007). Both the pathogen and the host influence the success of cyst germination and consequently infection (WALKER and VAN WEST 2007). For example, zoospores from different oomycete species, such as *Pythium* and *Phytophthora*, encyst differently, and spores germinate more easily on dicotyledenous plant species than monocotyledenous plants (WALKER and VAN WEST 2007).

Phytophthora cinnamomi cyst germination involves penetration of plant tissue by the germ tube (HARDHAM 2005; LATIJNHOUWERS *et al.* 2003; VAN WEST *et al.* 2003). Plant penetration can occur in the region between epidermal cells, or by piercing the outer cell wall. Swellings at the tips of germtubes, known as appressoria, are responsible for penetration of the germtube through cell walls (HARDHAM 2005; HARDHAM 2007; LATIJNHOUWERS *et al.* 2003). Germtubes penetrating along the anticlinal wall often do not form appressoria, while germtubes that penetrate the periclinal wall commonly have appressoria (HARDHAM 2001a). Infection and host colonization also relies on production and secretion of degradative enzymes for breaking down physical plant barriers such as cell walls (HARDHAM 2007). Host nutrients are used as energy sources for the colonization of the host by the pathogen's mycelia early in the infection process. Haustoria, structures responsible for nutrient accumulation, form in the cortical cells of roots by degradation of the plant cell wall, after which the plant's cell wall is invaginated by the pathogen cell wall (HARDHAM 2007). At later stages in the infection cycle, usually two to three days after infection, the pathogen starts to sporulate. Chlamydospores are produced inside cortical cells, and sporangia, the source of

new zoospores, on the root surface, repeating the life cycle of the pathogen upon the plant's death (HARDHAM 2001b; VAN WEST *et al.* 2003).

Pathogenicity factors

Oomycetes use secreted pathogenicity factors and effectors to manipulate host cells and host defence responses to facilitate infection (BIRCH *et al.* 2008; KAMOUN 2006; LAMOUR *et al.* 2007). Successful plant infection by oomycetes and fungi rely on efficient spore production followed by dispersal and successful penetration (LATIJNHOUWERS *et al.* 2003). The long term outcome of infection (successful establishment of plant disease or a failure to spread throughout and colonize the plant) depends on the specific interaction between the host and the pathogen at a molecular level. If a pathogen is capable of evading plant immune responses and manipulating host processes it will be able to establish disease.

A large number of pathogenicity proteins, which aid the successful establishment of plant disease, are encoded in pathogen genomes (KAMOUN 2006; LAMOUR *et al.* 2007). These include enzymes involved in degrading host cell walls, proteins that protect against reactive oxygen species (ROS) and proteins involved in signal transduction and communication (KAMOUN 2006). Cell wall degrading enzymes (CWDEs) break down host cell walls, allowing for pathogen entry (HAVE *et al.* 2002). Examples of CWDEs include polygalactorunases, cellulases, glucanases, glucosidases and cutinases. Once the pathogen has broken down the host's protective barrier, it can penetrate and colonize host tissue. Proteins involved in protection against ROS, such as superoxide dismutase, neutralize superoxide and hydrogen peroxide (NEBERT and VASILIOU 2004), while others such as ATP binding cassette (ABC) transporters export compounds that might be detrimental to the pathogen (DASSA and BOUIGE 2001). Guanosine nucleotide-binding proteins (G-proteins) such as mitogen-activated protein kinases (MAPK) are membrane bound receptors which transmit extracellular signals into cells (LATIJNHOUWERS *et al.* 2003; LI *et al.* 2007). MAP-kinases have been linked to appressoria formation, pathogenesis and colonization.

Oomycete effectors can be divided into two categories, apoplastic and cytoplasmic effectors (KAMOUN 2006). Apoplastic effectors are secreted into the extracellular spaces to interact with their targets outside the cell. One of the most abundant classes is the enzyme inhibitors, which protect against the hydrolytic activity of plant Pathogenesis Related (PR) proteins such as glucanases and cellulases (MISAS-VILLAMIL and VAN DER HOORN 2008). The Nep 1-like proteins (NLPs), which function by triggering necrosis during the necrotrophic phase of

infection is another example of apoplastic effectors (PEMBERTON and SALMOND 2004). Cellulose Binding Elicitor and Lectin-like (CBEL) proteins are involved in necrosis, and also play an important role in attachment to plant surfaces (VILLALBA *et al.* 1997).

Cytoplasmic effectors are secreted into host cells by haustoria or infection vesicles, and are involved in gene-for-gene interactions with plant R (resistance) proteins and thus race-specific resistance (KAMOUN 2006). The two major classes of cytoplasmic effectors in oomycetes are RXLR (Arginine-any amino acid-Leucine-Arginine) and Crinkling and necrosis-inducing (CRN) proteins. RXLR effectors have conserved N-termini containing a signal peptide and the RXLR motif, with highly variable C-terminals, which contain the effector domain, and are associated with biotrophic oomycete pathogens (BIRCH *et al.* 2008; STASSEN and VAN DEN ACKERVEKEN 2011). CRN proteins also possess a motive (Leucine-Phenylalanine-Leucine-Alanine-Lysine) and a signal petide on the N-terminal, and as the name suggest, are associated with necrotrophic pathogens (STAM *et al.* 2013).

Recently, the Illumina sequencing platform was used to produce a RNA-seq library of the cyst and germinating cyst stages of *P. cinnamomi* (REITMANN 2013). Approximately 2000 unigenes with putative roles in pathogenesis were identified from a total of 70 000 unigenes. Enzymes involved in cell wall degradation and protection against oxidative stress such as reductases, glucanases, glucosidases and polygalacturonodases were most common, and 46 putative RXLR effectors, elicitins and necrosis inducing proteins were also identified (REITMANN 2013).

The relative transcript abundance of three effector genes, one CWDE and three genes involved in pathogen adhesion were investigated in mycelia, sporulating mycelia, zoospores and cysts and germinating cysts by Reverse Transcriptase quanitative PCR (RT-qPCR) (REITMANN 2013). The three classes of effector transcripts investigated included transcripts corresponding to a RXLR gene, an elicitin gene and a necrosis-inducing protein. The RXLR and elicitin transcripts showed an increased abundance in zoospores and germinating cysts when compared to their abundance in mycelia, implicating their involvement in early infection. The expression of necrosis-inducing *Phytophthora* protein 1 (NPP1) transcripts was upregulated in zoospores. The transcripts of a polygalactorunase were present at the highest levels in zoospores, confirming its pectin-degrading role in early infection.

The examples of genes involved in pathogen adhesion investigated included a CBEL gene, an adhesion-like gene and a mucin-like gene. Transcripts corresponding to the CBEL and adhesion-like gene were induced in zoospores, while those of the mucin-like gene were upregulated in mycelia and sporulating mycelia (REITMANN 2013). Induction of CBEL and

adhesion-like genes in zoospores corresponds to their roles in cell wall deposition and adhesion, facilitating attachment of zoospores to host surfaces. In contrast, the abundance of mucin-like transcripts in mycelia and sporulating mycelia prompted the author to hypothesize that mucins, which have proposed roles as lubricants, protectants and signal peptides (HICKS *et al.* 2000), are involved in protection, as opposed to pathogen adhesion.

From its life history traits it is clear that species within the genus *Phytophthora* are highly adapted for their role as plant pathogens (GOODWIN 1997). Sexual oospores allow for the survival of the organism in the absence of hosts or in unfavourable environmental conditions, and zoospores mediate long distance dispersal (GOODWIN 1997), all contributing to making this species hardy, proliferate and efficient. The ability of *P. cinnamomi* to rapidly disperse through plant material and soil, its manipulation of host cells through pathogenicity factors and the large negative impact it has on food crops, ecosystems and biodiversity highlights the importance of research on this very successful pathogen (HARDHAM 2005).

Persea americana (Mill.) and the avocado industry in South Africa

Persea americana is an economically important nutritious fruit crop that has been grown by mankind for roughly 9000 years (WHILEY *et al.* 2002). Evidence found in the avocado's centre of origin in Meso-America (Mexico, Guatamala and Central America) suggest that Mayan and Aztec civilizations already selected for traits such as fruit size in the earliest days of avocado production. Today, in the 21st century, scientists are still selecting for scion traits such as fruit size and quality, smaller trees with sustainable yield, and for rootstock traits such as root rot resistance and tolerance to salt and calcium carbonate in soil (WHILEY *et al.* 2002).

Persea americana belongs to the *Lauraceae* family, and this polymorphic species is divided into three botanical varieties: the Mexican (var *drymfolia*), Guatamalan (var *guatamalensis*) and West-Indian (var *americana*) races (WHILEY *et al.* 2002). As the names suggest, the Mexican race originated in Mexico, and the Guatamalan race in Guatamala, but the West-Indian race originated on the Pacific coast of Central-America. Confusion generated by the term "West-Indian" led to the renaming of the West-Indian race to the Lowland race, but the original term is still widely used (WHILEY *et al.* 2002). Most cultivars used in commercial horticulture are hybrids that arose through crosses between the three different races. "Hass" and "Fuerte", the most widely used scion cultivars, are the products of crosses between

Mexican and Guatamalan varieties, while tropical cultivars are hybrids between Guatamalan and West-Indian races (WHILEY *et al.* 2002).

The first introduction of avocado into South Africa occurred in the 1800's, and consisted of West-Indian seedlings cultivated in the Durban region of KwaZulu-Natal (WHILEY *et al.* 2002). However, the fruit from those first orchards were of poor quality, which led to the introduction of Mexican and Guatamalan seedlings into South Africa in the 1920's. These trees were better suited to the climatic conditions encountered in South Africa, and signalled the start of avocado production in South Africa (WHILEY *et al.* 2002).

Production

Approximately 91 603 tons of avocados were produced on 16 350 ha of commercial avocado orchards in South Africa in 2012, with an average yield of 56 026 hectograms/hectare (faostat.fao.org). Production is concentrated in the warm, subtropical regions of Limpopo and Mpumalanga, but KwaZulu-Natal with its lower latitude and resulting cooler temperatures also contributes a small portion of the annual production of avocados. Of the 90 000 tons produced each year, 40 000 tons are exported to the United Kingdom and other European countries, while 10% of the annual crop is processed into purée and avocado oil (DONKIN 2007). In the past, the avocado season in South Africa ran from March to September, and climatic differences between the northern growth regions and KwaZulu-Natal ensured that most cultivars were available throughout the season due to different harvest times in the different regions (DONKIN 2007). However, the availability of new avocado cultivars such as Carmen Hass guarantees year-round avocado production by Westfalia Technological Services (WTS) in Tzaneen.

The South African avocado industry expanded rapidly from the 1970s until 2003, but in recent years planting of new trees has declined (DONKIN 2007). The two major scion cultivars produced in South Africa are "Hass" and "Fuerte", although other cultivars such as 'Ryan' and 'Pinkerton' are also planted to a smaller extent (DONKIN 2007). Export markets' preference for the "Hass" cultivar has resulted in its wide-spread use in commercial South African orchards, and avocado nurseries, which supply trees for the establishment of new orchards as well as for the replacement of older orchards, also produce "Hass" trees to a greater extent.

Orchards that were established in the 1980s consist of the "Hass" scion cultivar grafted on the *Phytophthora*-tolerant rootstock "Duke 7" (DONKIN 2007). The "Hass" scion cultivar,

which reaches maturity later than “Fuerte” was selected in California in the early 1930s based on its fruit quality and high yield (WHILEY *et al.* 2002). The rootstock “Duke 7” was also selected in California in the 1950s for its moderate *P. cinnamomi* resistance (WHILEY *et al.* 2002). Today, however, the majority of trees are grown on the rootstock Dusa® (“Merensky II”), which is more tolerant to PRR than “Duke 7”. “Bounty” and “Velvick” are also occasionally used. Once a superior tolerant rootstock is identified after extensive field trials, the rootstock often serves as the nurse seedling rootstock for clonal propagation (BEN-YA'ACOV and BET-DACON 1985).

Disease control

A number of bacteria, fungi and oomycetes cause foliar, fruit and soilborne diseases on avocado, leading to reductions in crop yield and quality (WHILEY *et al.* 2002). Examples include cankers caused by the bacterium *Pseudomonas syringae*, *P. cinnamomi* and *Phytophthora heveae* Thompson, root rots caused by *P. cinnamomi* and *Armillaria* spp. and Cercospora spot on fruit, stems and leaves caused by *Pseudocercospora purpurea* (Cooke) Deighton (WHILEY *et al.* 2002). Amongst these, PRR and white root rot, caused by *Rosellinia necatrix* Berl. ex Prill are the most severe, and leads to devastating losses in the global avocado industry each year. At the moment, white root rot is only causing significant damage to orchards in Spain and Israel (PLIEGO *et al.* 2009), while PRR is considered a serious problem world-wide.

Fungicides targeting pathogen cell walls are not effective against oomycetes such as *P. cinnamomi,* due to the differences in the cell walls of oomycetes and fungi. Others, such as meatalaxyl, which targets RNA polymerases have been successfully used against some *Phytophthora* spp. (LATIJNHOUWERS *et al.* 2003). Phenylamides and phosphonates (phosphite) are both systemically translocated fungicides used to control *P. cinnamomi* (GUEST and GRANT 1991; HARDY and BARRET 2001). Phenylamides are translocated in the xylem, while phosphites are translocated in both the xylem and the phloem (GUEST and GRANT 1991), and in recent years the use of phosphonates against root rot has dominated the industry (HARDHAM 2005).

Upon administration, the chemicals are taken up into the tissues of the tree through its phosphonate pathways (GUEST and GRANT 1991; HARDY and BARRET 2001). Avocado trees are not able to metabolize phosphonates, and thus the chemical remains in the tree tissue for a prolonged period of time. Despite the wide-spread use of these chemicals, the exact

mode of action of phosphite is still unclear (GUEST and GRANT 1991). A combination of the direct inhibition of the pathogen and the up-regulation of the host response is most probable (DALIO *et al.* 2014; KING *et al.* 2010). Researchers believe that phosphite may prevent the oomycete from producing suppressor molecules, or stimulate the production of elicitor molecules allowing the host to recognize the pathogen and to initiate a response against it (GUEST and GRANT 1991). In a study conducted by Wilkenson *et al.* (2001a) the authors observed that phosphonate treatment significantly reduced the number of zoospores associated with the *P. cinnamomi* infection. Even if treatment is not able to completely eliminate the pathogen, but is able to decrease the load of the infection, it will result in improved tree health (HARDY and BARRET 2001).

The effectiveness of phosphite treatment has been shown to vary between isolates of the same species, as well as between different environments (GUEST and GRANT 1991). Treatment can also have toxic effects on some plants, with symptoms ranging from foliar damage to a reduction in root growth (BARRET *et al.* 2002; FAIRBANKS *et al.* 2001; FAIRBANKS *et al.* 2002; NARVARANANT *et al.* 2004). Careful management of dosages used for treatment and application can limit toxicity in sensitive plants.

A common concern when using chemical agents to manage phytopathogenic fungi and oomycetes is the evolution of resistance or a decreased sensitivity to the chemical control agent. In a study by Dobrowolski *et al*. (2008) the authors showed that *P. cinnamomi* isolates from avocado orchards that had previously been treated with phosphite were better able to colonize phosphite-treated plant tissue compared to isolates from untreated avocado orchards or native vegetation. No significant difference in colonization ability was observed between isolates from treated and untreated sites on untreated plant material. None of the isolates used in the study were resistant to phosphite, but the enhanced colonization ability of isolates previously exposed to phosphite treatment indicates that this treatment may select for decreased phosphite sensitivity in the population (DOBROWOLSKI *et al.* 2008).

Compost and mulches are often worked into the soil to improve drainage, as moisture plays a very important role in the establishment, spread and severity of the infection (HARDHAM 2005; REEKSTING *et al.* 2014). Mulching mimics avocado's native habitat, South American forests, where a thick layer of organic material is found on the forest floor. This layer of organic litter is rich in micro-organisms, and fulfils many functions. The presence of healthy levels of micro-organisms results in high levels of external cellulases, decreasing *P. cinnamomi* inoculum through hydrolysis of its cellulose-rich cell walls. The dead plant material provides mineralized nutrients, and serves as a buffer between the roots in the soil

and the external environment, protecting the roots from drastic environmental changes (WHILEY *et al.* 2013).

An integrated approach combining tolerant rootstocks, chemical agents and compost have proved most effective in limiting the severity of the impact of *P. cinnamomi* on tree health and crop yield. Together, these three approaches succeed in reducing the suitability of the ecological environment to *P. cinnamomi*. The three most important factors that should be considered for long term control of this disease is thus reliable detection and diagnosis of *P. cinnamomi*, the responsible use of chemical control, good management of general orchard health and finally, breeding of resistant rootstocks (HARDHAM 2001b).

In order to combat this serious disease problem of avocado production, a sound understanding of the interaction between *P. cinnamomi* and avocado is required. As discussed previously, high throughput sequencing has become one of the most important tools to study pathogens such as *Phytophthora* through analysis of their genomic and transcriptomic data. Another invaluable tool, which often complements sequencing approaches, is transformation procedures for *Phytophthora* spp.

Transformation of oomycetes

The development of transformation systems for many oomycetes in the last 20 years has served as useful tools to study components of the biology of *Phytophthora* spp., such as gene function through gene disruption (FINCHAM 1989), which researchers were previously unable to. After Judelson developed a protocol for transformation of *P. infestans* using antibiotic resistance genes as markers (JUDELSON and MICHELMORE 1991; JUDELSON *et al.* 1991), species of *Phytophthora* were transformed mainly with fluorescent protein genes such as GFP and mCherry. Fluorescent protein genes were useful markers for optimizing transformation of *Phytophthora* spp., and allowed for *in planta* investigation of host-pathogen interactions. With the availability of genome sequences for a number of *Phytophthora* spp. (HAAS *et al.* 2009; LAMOUR *et al.* 2012; TYLER *et al.* 2006), and the shift to research on pathogenicity factors in the *Phytophthora* community in recent years, the ability to routinely transform oomycetes has become important in order to experimentally validate sequence data by functional characterization of pathogenicity genes. To date, stable transformation has been reported in oomycete genera such as *Achlya*, *Phytophthora*, *Saprolegnia* and *Pythium* (JUDELSON and AH-FONG 2009).

Methods for transformation

Genetic engineering of microorganisms relies on the successful uptake of exogenous DNA into the nucleus of the recipient cell, and expression and stable maintenance of foreign genes in the host cells (RUIZ-DÍEZ 2002). For this, a vector containing the gene of interest, control elements (promoters and terminators) to direct expression and efficient transformation and selection systems are required (JUDELSON and AH-FONG 2009; WINK 2011). Techniques for the successful transformation of oomycetes were only developed in the early 1990's (JUDELSON and MICHELMORE 1991), more than ten years after the first transformation of a fungus (CASE *et al.* 1979). Some transformation methods are described in the following sections, and their advantages and disadvantages highlighted **(Table 1)** (MEYER 2008).

Polyethyeleneglycol (PEG) - mediated protoplast transformation

PEG-mediated protoplast transformation is most commonly used in the transformation of oomycetes. A large number of *Phytophthora* spp. such as *P. parasitica* (BOTTIN *et al.* 1999; LE BERRE *et al.* 2008a), *P. palmivora* (VAN WEST *et al.* 1999b), *P. sojae* (CHEN *et al.* 2009), *P. infestans* (JUDELSON and MICHELMORE 1991; SI-AMMOUR *et al.* 2003), *P. brassicae* (SI-AMMOUR *et al.* 2003), *P. capsici* and *P. ramorum* (RIEDEL *et al.* 2009) have been transformed using this method. This method relies on the use of enzymes to degrade the cell walls of mycelial cells to produce competent protoplasts able to take up foreign DNA (DAVEY *et al.* 2005; RADLEDGE and KRISTIANSEN 2001).

The mycelia (or cells) are first washed in a hypertonic solution (such as a mannitol based wash buffer) to stimulate the retraction of the plasma membrane from the cell wall (DAVEY *et al.* 2005). Hydrolytic enzymes, such as β-1,3-glucanases and chitinases are then used to degrade the cell walls. After removal of mycelial cell walls, protoplasts are osmotically stabilized in hypertonic mannitol, sorbitol, magnesium sulphate or sodium chloride solutions, which prevent the cells from bursting (BARTNICKI-GARCIA and LIPPMAN 1966; RADLEDGE and KRISTIANSEN 2001). The resulting protoplasts are competent to take up foreign DNA (RUIZ-DÍEZ 2002), and transformation of the protoplasts is performed in the presence of $CaCl_2$ and PEG, which stimulate DNA uptake and induces clumping of protoplasts, in order to trap DNA, respectively (FINCHAM 1989; RADLEDGE and KRISTIANSEN 2001; RUIZ-DÍEZ 2002). Protoplasts are subsequently regenerated on an osmotically-buffered selection medium, which allows for the growth of transformants with the selectable marker. A lower

concentration of osmotic stabilizer in the medium can increase transformation efficiencies in some cases, as high concentrations can inhibit protoplast regeneration and growth (RUIZ-DÍEZ 2002). The production of viable protoplasts, as well as uncertainties regarding vectors and selection of transformants were the main problems that had to be overcome to enable protoplast transformation of oomycetes (JUDELSON 1996).

In a study by Bartnicki-Garcia and Lippman (1966) the authors used an extracellular enzyme solution consisting mainly of β-glucanases to release protoplasts from *P. cinnamomi* and *P. parasitica*. Young, actively growing colonies for enzyme digestion were obtained by homogenizing mycelia and subsequently inoculating broth. The authors observed a large degree of variation between different experiments. In some experiments, large numbers of protoplasts were obtained, and in others none. They identified two main mechanisms by which protoplasts are released from hyphae. At earlier points in the digestion, the cytoplasm is usually released through a pore in the membrane, which is known as budding. At later stages in the process, protoplasts are formed by sealing off swollen cells **(Figure 5)**. The authors also observed that the protoplasm present within the protoplasts differed depending on the cells from which they originated. In some instances, protoplasm was discharged from the cell without being delimited by a cell membrane, which is usually only observed when digestion in the absence of stabilizers.

Zoospore electroporation

Electroporation has been used to transform bacteria, fungi (both filamentous and yeasts), animal cells and plant protoplasts. Using this method, short, high amplitude electric shocks are used to render membranes permeable through the formation of pores, which facilitates DNA uptake by the recipient cells, resulting in transformation (RUIZ-DÍEZ 2002). Transformation of oomycetes through zoospore electroporation eliminates the need for cell wall manipulations associated with the release of protoplasts (JUDELSON and AH-FONG 2009). The major disadvantage associated with zoospore electroporation is the large numbers of zoospores that are required (HUITEMA *et al.* 2011). This procedure is thus not feasible in species such as *P. cinnamomi* which sporulate poorly.

A major determining factor in the success of zoospore electroporation is the health of zoospores (HUITEMA *et al.* 2011). Zoospores that are actively swimming around serve as the best starting material. Zoospores are released from sporangia, and LiCl is added to prevent encystment (JUDELSON and AH-FONG 2009). Zoospores are then concentrated on a density

gradient, followed by the application of the electric field. The voltage of the electric field and other ionic conditions should be optimized for each species, to ensure the introduction of the transgene, while at the same time maintaining viability of zoospores (JUDELSON and AH-FONG 2009). Following electroporation, regeneration media is added to the zoospore suspension in order to allow zoospores to germinate before they are plated onto selectable media (HUITEMA *et al.* 2011).

Zoospore electroporation of *P. infestans* zoospores typically results in more transformants when compared to protoplast transformation, but Latijnhouwers and Govers have reported that gene silencing is less effective in transformants obtained in this manner (LATIJNHOUWERS and GOVERS 2003). This could be due to less copies of plasmid DNA integrating (JUDELSON and AH-FONG 2009), corresponding to a study by Ah-Fong *et al*. (2008) in which the authors found that transformation of protoplasts with silencing constructs resulted in twice as many transformants when compared to zoospore electroporation (AH-FONG *et al.* 2008). A study by Blanco and Judelson (2005) also found that transformation of protoplasts with silencing constructs resulted in higher numbers of silenced *P. infestans* strains when compared to transformation by zoospore electroporation (BLANCO and JUDELSON 2005).

Microprojectile bombardment

This method is used to introduce foreign DNA into cells without the manipulation of cell walls, which is often the cause of difficulties experienced with transformation of plants, fungi and oomycetes (WINK 2011). The second advantage of a biolistics approach is that any organism (plant, fungus or oomycete) and more importantly, tissue type can be transformed, and that relatively small amounts of starting material is required (JUDELSON and AH-FONG 2009). Species which do not sporulate or grow well in culture can thus be transformed by eliminating the need for the production of germinating cysts (JUDELSON 1996).

The expression cassette used for transformation is amplified in *Escherichia coli* prior to transformation, after which the cassette is excised from the vector, and bound to tungsten or gold particles (WINK 2011). Large fragments are prone to shearing, thus the size of expression cassettes should be as small as possible. Tungsten or gold particles are coated with DNA through precipitation with $CaCl_2$ and spermidine (WINK 2011). Air pressure is used to accelerate the particles, and the particles are ‘shot’ into cells. In those cells that survive the impact, the DNA is integrated into the genome by recombination processes within the

cell. The pressure, and thus force behind the particle can be adjusted to reach specific tissues, or to permeate cell walls that are very thick (WINK 2011). Particle sizes are generally 1-2 µm, with a density of 19 g/cm^3, and a particle speed of 440 m/s is sufficient for transformation of most tissues (WINK 2011).

The success rate for transformation by microprojectile bombardment is between 1-5 %, and variation in expression levels often occur (WINK 2011). Cvitanich and Judelson (2003) successfully transformed *P. infestans* sporangia, germinating cysts and mycelia with a construct containing neomycin phosphotransferase (npt) as selectable marker and the *β-glucuronidase* (*GUS)* reporter gene by microprojectile bombardment. The protocol was optimized in terms of target tissue, bombardment pressure, distance and selection, after which an average of 14 transformants/µg of DNA (1 shot, using 10^6 sporangia) was obtained (CVITANICH and JUDELSON 2003).

There are several advantages associated with transformation by microprojectile bombardment. Protoplasting is not required, and different target tissues such as sporangia, germinating cysts and mycelia can be used (CVITANICH and JUDELSON 2003). For PEG-mediated protoplast transformation only freshly germinated spores can be used as starting material. The maximum rates of obtaining transformants is similar for microprojectile bombardment and PEG-mediated protoplast transformation, but a greater level of variation in transformation rates is observed for PEG-mediated protoplast transformation (CVITANICH and JUDELSON 2003). Microprojectile bombardment also allows for higher through put rates, as more than 20 shots can be performed per hour (CVITANICH and JUDELSON 2003). A disadvantage of this method is the relatively high number of heterokaryons obtained, but this shortcoming can be overcome by plating zoospores from primary transformants on selectable medium to obtain homokaryons (CVITANICH and JUDELSON 2003; JUDELSON and AH-FONG 2009).

Agrobacterium tumefaciens-mediated transformation

Agrobacterium tumefaciens and *Agrobacterium rhizogenes* are soil-borne bacteria that are routinely used for the introduction of foreign DNA into plant cells (WINK 2011). Both species contain plasmids which are approximately 200 kb in size, and contain 25 genes mainly involved in virulence (*vir* genes), the transfer of bacterial DNA into the genome of the plant and oncogenes. *Agrobacterium tumefaciens* is used more widely due to the fact that it

infects above ground plant organs, causing crown gall, while *A. rhizogenes* infects roots, causing uncontrolled proliferation known as hairy root (WINK 2011).

The Ti (tumour-inducing)-plasmid of *A. tumefaciens* contains a smaller DNA fragment of approximately 20 kb known as the T-DNA (transfer DNA) (WINK 2011). The T-DNA portion of the Ti-plasmid is integrated into the host's genome. The T-DNA encodes oncogenes and genes encoding amino acid derivatives such as opines, auxins and cytokinins, and is flanked by 25 bp short imperfect direct DNA repeats known as the left border (LB) and right border (RB) (VIJN and GOVERS 2003; WINK 2011). The flanking regions serve as the recognition sites necessary for the excision of the T-DNA from the Ti-plasmid, for binding of proteins involved in transporting the T-DNA into the host's nucleus, and the subsequent integration into the host's genome (WINK 2011).

A binary system in which two different plasmid vectors are present in *Agrobacterium* is used for the transformation of plants, yeasts and filamentous fungi (WINK 2011). The binary vector contains the T-DNA, with left and right borders, a multiple cloning site (MCS) for insertion of the foreign gene and a selectable marker, and an origin of replication (ORI) on the backbone of a bacterial plasmid such as pBR322. The maximum size of the insert DNA is 150 kb, and integration of the foreign DNA into the genome of the host is controlled by the genes responsible for virulence and integration on the disarmed Ti-plasmid. The disarmed Ti-plasmid also contains a selectable marker providing a selectable advantage to ensure that the plasmid is maintained in *Agrobacterium* (WINK 2011).

Vijn and Govers (2003) were able to successfully transform *P. infestans* with the *GUS* reporter gene using *Agrobacterium*-mediated transformation. A construct with a T-DNA carrying the *npt* gene under the control of the *hsp70* promoter and *ham34* terminator from *Bremia lactuceae* was first used to confirm that *Agrobacterium* T-DNA is incorporated into the genome of *P. infestans* (VIJN and GOVERS 2003). After confirmation of integration of the foreign gene, *P. infestans* strains were transformed with a vector containing a T-DNA consisting of the *GUS* reporter gene under the control of *ham34* promoter and terminator elements, and the *npt* selectable marker (VIJN and GOVERS 2003).

There are several advantages associated with *Agrobacterium*-mediated transformation. The production of protoplasts, the major obstacle using the PEG method is circumpassed. This method appears to be less labour-intensive and more efficient, and appears to result in the incorporation of one or two copies of the gene, with multiple copies often inserting during PEG-mediated protoplast transformation (VIJN and GOVERS 2003). However, a disadvantage

of *Agrobacterium*-mediated transformation is the limited host range of the bacterium, which limits its use to only its host species (WINK 2011).

The first successful transformation of an oomycete

Phytophthora infestans, the causal agent of light blight of potato and tomato was the first oomycete that was stably transformed with foreign genes (JUDELSON and MICHELMORE 1991; JUDELSON *et al.* 1991). Non-pathogenic fungi such as *Saccharomyces cerevisiae* Gasperini, *Neurospora crassa* Shear and B. O. Dodge and *Aspergillus nidulans* (Eidam) G. Winter were the first fungi to be transformed successfully (FINCHAM 1989). This was followed by the successful transformation of pathogenic asco- and basidiomycetes. Attempts to transform oomycetes with vectors and protocols developed for fungi were not successful, and were attributed to the distant phylogenetic relationship between fungi and oomycetes (FINCHAM 1989; JUDELSON *et al.* 1991).

Judelson *et al.* (1991) transformed *P. infestans* with the bacterial *hygromycin phosphotransferase (hpt)* gene (confers resistance to hygromycin B) and the *npt* gene (confers resistance to geneticin/G418). Each gene was expressed under the direction of two different promoter combinations in two separate experiments. In the first, the *hsp70* heatshock promoter was teamed with the *ham34* terminator from *B. lactuceae* (lettuce downy mildew), and in the second experiment, the *hsp70* promoter was replaced with the *ham34* promoter, also from *B. lactuceae* (JUDELSON *et al.* 1991).

Two different methods were used to introduce the plasmid vectors into the protoplasts in the above mentioned study. Introduction by cationic liposomes involved the incubation of the DNA with Lipofectin reagent before addition to the protoplasts. For transformation without liposomes, plasmid DNA was mixed with carrier DNA (salmon) before addition to the protoplasts (JUDELSON *et al.* 1991). The protoplast suspensions of both methods were subsequently treated with PEG and $CaCl_2$ to mediate transformation, followed by protoplast regeneration and plating on selection plates for identification of putative transformants.

Stable transformants were obtained for all combinations of bacterial marker genes, promoters and transformation protocols (JUDELSON *et al.* 1991). However, the transformation efficiency was significantly lower compared to transformation efficiencies reported in fungi. The liposome method yielded a greater number of transformants (12, in comparison to two obtained from the carrier DNA method). Addition of homologous *P. infestans* sequences to the vectors did not increase transformation efficiency, and the authors concluded that the

low protoplast regeneration rate might be the cause of the low transformation efficiencies, and subsequent experiments confirmed that the transgenic strains retained pathogenicity.

Transformation of *P. cinnamomi*

Only two reports on the transformation of *P. cinnamomi* is present in the literature (BAILEY *et al.* 1993; HORTA *et al.* 2008). In the first, intact mycelia of *P. cinnamomi* was transformed with either the *GUS* or *hptII* genes through microprojectile bombardment (BAILEY *et al.* 1993). Expression of the *hptII* gene was driven by a *hsp70* promoter form *Ustilago maydis* (DC) Corda, and putative transformants were identified following selection on V8 agar plates containing 400 µg/ml of hygromycin B (BAILEY *et al.* 1993). A cauliflower mosaic virus 35S constitutive promoter (CaMV35S) directed the expression of the *GUS* gene. Integration of the *GUS* gene was confirmed by histochemical and fluorometric GUS assays. Heterokaryons were eliminated by production of single zoospore derivatives of transformants, and southern blots were used to confirm integration of plasmid DNA (BAILEY *et al.* 1993).

In the second example, a PEG and $CaCl_2$ -mediated protoplast transformation with silencing constructs was used to silence a *β-cinnamomin* gene in *P. cinnamomi* (HORTA *et al.* 2008). Following silencing of the *β-cinnamomin* gene, RT-qPCR analysis of the expression of other elicitin genes showed reduced transcript levels of other elicitin genes in the cluster. The *P. cinnamomi* strain in which the *β-cinnamomin* gene was silenced showed a reduced growth rate on *Quercus suber* roots, leading to the authors hypothesizing that elicitins are involved in pathogenicity (HORTA *et al.* 2008). The limited amount of literature describing transformation experiments in *P. cinnamomi* complicates the establishment of transformation systems for *P. cinnamomi*, as a limited pool of information can be drawn upon.

Optimizing protoplast production and regeneration

Major limitations of PEG-mediated protoplast transformation include the large amounts of biological material required and low protoplast regeneration rates, which ultimately results in limited numbers of transformants. The development of a protocol which would enable the production of larger numbers of protoplasts, and higher protoplast regeneration rates would significantly increase transformation efficiency (MCLEOD *et al.* 2008). McLeod *et al.* (2008) adapted a protocol used for the production of *Arabidopsis* protoplasts for use in

Phytophthora and *Pythium* to improve transformation efficiencies in *Phytophthora* spp. and other oomycetes.

Modifications were made to the enzymes used for degradation of the cell walls, and the osmoticums used for digesting cell walls and washing of protoplasts, which improved the buffering capacity and protoplast stability respectively **(Figure 6)** (McLeod *et al.* 2008). The PEG solution in the new protocol contained mannitol (which is absent in most other protocols), which the authors found contributed to the stability of the protoplasts. The $CaCl_2$ concentration was also increased, to maximize DNA uptake, and the amount of mannitol in the regeneration medium was decreased (McLeod *et al.* 2008).

Less starting material is required for the modified *Arabidopsis* protocol, the tissue survival rate is higher in comparison with other methods, and protoplast regeneration rates are similar to those achieved using the protocol of Judelson *et al.* (1991). The authors found the *Arabidopsis*-based method more suited for use in oomycetes, and concluded that this may be due to the fact that oomycetes are more closely related to plants than to fungi (McLeod *et al.* 2008). Protection of transforming DNA through the use of carrier DNA also appears to increase the number of successful transformants (Mort-Bontemps and Fèvre 1997). In experiments conducted by Mort-Bontemps *et al.* (1993) transformation rates increased in response to increasing amounts of salmon DNA. The addition of 600 µg of salmon DNA to 20 µg of plasmid DNA resulted in a four-fold increase in the number of transformants

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Examples and applications of pathogens transformed with Fluorescent Proteins

Besides the usefulness of using fluorescent protein genes as reporter systems to optimize transformation procedures, fluorescently tagged pathogens have many applications. These include quantitative *in planta* detection of pathogens, the ability to detect pathogens in parts of the plant where their abundance is low, pathogen detection before the onset of visible disease symptoms, the study of plant-microbe interactions and the tracking of genetically modified strains. Examples of different studies using fluorescently-tagged pathogens are discussed in this section.

Studying the development of the pathogen and its interaction with the host *in planta*

Bottin *et al.* (1999) successfully transformed *P. parasitica* var *nicotianae,* a root pathogen colonizing tobacco, with a plant-adapted *GFP* gene (sGFP(S65T)) by PEG-mediated protoplast transformation in the presence of Lipofectin. Transformants were identified by selection for hygromycin resistance, followed by examination of transformants for fluorescence under a microscope (BOTTIN *et al.* 1999). Researchers observed a high frequency of co-transformation, as the *sgfp* gene was present in every transformant, with some transformants in possession of multiple tandemly incorporated copies of the pAHb6 vector in their genome (BOTTIN *et al.* 1999).

The authors subsequently used a GFP-tagged *P. parasitica* var *nicotianae* strain for *in vitro* and *in planta* detection of the pathogen. The development of hyphae into sporangia, the release of zoospores from sporangia, zoospore encystment and germination was easily observed as fluorescence was maintained throughout all developmental stages. Germinated sporangia and cysts were readily observed on the root surfaces of tobacco plants infected with the transformed strain, while hyphal growth was observed within root and leaf material (BOTTIN *et al.* 1999).

In a similar study, Pliego *et al.* (2009) investigated the infection process of avocado by the ascomycete fungus *R. necatrix*, the causal agent of white rot in avocado, using a transgenic *R. necatrix* strain expressing GFP **(Figure 7)**. Six-month old avocado roots were inoculated with transgenic *R. necatrix*, and root material was analyzed one month post-infection using Confocal Laser Scanning Microscopy (CLSM) (PLIEGO *et al.* 2009). Plant invasion was found to occur at different positions in the crown region simultaneously. Penetration also occurred through the lenticels, and in the regions between epidermal cells, after which the infection spread through the epidermal and cortical root cells into the primary and secondary xylem (PLIEGO *et al.* 2009). Penetration of the pathogen through the root tip or along the secondary roots was absent. The ability to identify all tissues colonized by *R. necatrix* during infection enabled the researchers to conclude that the infection strategy of *R. necatrix* differs from that of other plant pathogenic fungi.

By tagging *P. sojae* with GFP, Chen *et al.* (2009) were able to show that the germ tubes of transgenic *P. sojae* were longer and thinner in the roots of resistant soya plants, compared to shorter, thicker germ tubes in susceptible plants (CHEN *et al.* 2009). Their study was the first to report the use of laser microscopy to identify differences between resistant and susceptible soya cultivars, and the results were similar to reports for different potato cultivars

and *P. infestans* (LAPWOOD 1968). Tagged pathogens could thus contribute to the identification of resistance mechanisms in plants.

In planta pathogen detection before the onset of obvious external disease symptoms

An experiment by Le Berre *et al.* (2008) confirmed the use of GFP-tagged pathogens in monitoring infection *in planta* before the onset of obvious external disease symptoms. A transgenic *P. parasitica* strain constitutively expressing GFP was used to infect tomato plants. One day after the addition of zoospores to the tomato roots the plants appeared healthy, with only a few mycelia observable around the roots. However, microscopic analysis of the roots confirmed the presence of the pathogen in host tissues **(Figure 8)** (LE BERRE *et al.* 2008a). At two days post inoculation, the plants still appeared healthy, but the pathogen was visible within the root hairs after microscopic examination, and staining with propidium iodide showed that the cell walls had been destroyed (LE BERRE *et al.* 2008a). Macroscopical symptoms of infection, such as wilting, only appeared at four days post-inoculation, and after four days post-inoculation, damage to the aerial parts of the plants occurred rapidly, with most of the plants dead at 10 days post-infection (LE BERRE *et al.* 2008a).

In planta pathogen quantification

Si-Ammour *et al.* (2003) used a double cassette plasmid vector to transform *P. brassiceae* and *P. infestans* with GFP. Comparisons between previous transformation protocols relying on the use of two plasmid vectors revealed that transformation using one vector containing both the selectable marker and non-selectable gene generated a higher number (three-fold increase) of transformants compared to co-transformations where the selectable marker is carried on another plasmid. A synthetic GFP with a S65T mutation and a higher GC-content was used, as it fluoresced stronger and more stably in comparison with the wild type (SI-AMMOUR *et al.* 2003).

The authors subsequently used the transgenic *P. brassiceae* and *P. infestans* to quantify induced resistance in potato and *Arabidopsis* in response to the application of chemical inducers of resistance. Induced resistance has previously been quantified by more subjective methods such as estimation of diseased area and sporulation (SI-AMMOUR *et al.*

2003). By quantifying fluorescence and measuring disease symptoms *in planta* the authors were able to conclude that β-aminobutyric acid (BABA) treatment prior to infection protected susceptible lines from infection **(Figure 9)**.

In planta evaluation of possible biocontrol agents

The use of biocontrol agents over chemical control measures has a number of advantages. Fungi and oomycetes often evolve resistance to chemical control agents due to the selective pressure placed upon them. Non-pathogenic strains of the same or other species can be used as biocontrol agents to decrease disease severity by competitive exclusion of the pathogenic strain.

Tagging of possible biocontrol and pathogenic strains with fluorescent markers allows for the visualization of the interaction between these two strains in the plant. In a study by Nonomura *et al*. (2001), three formae speciales of *Fusarium oxysporum*, namely *Fusarium oxysporum* f.sp *melonis* (pathogen), *Fusarium oxysporum* f.sp *radicis-lycopersici* (non-pathgen) and *Fusarium oxysporum* f.sp *fragariae* (non-pathogen) were transformed with a synthetic *GFP* gene to study the interaction between pathogenic and non-pathogenic strains in melon seedlings. The authors observed that the initial infection process was similar between the pathogenic and non-pathogenic strains. At five to seven days post-infection with the pathogenic strain, necrosis was observed in the roots surrounding the infection site. However, in plants also inoculated with non-pathogens, no root necrosis was observed (NONOMURA *et al.* 2001). This finding identified non-pathogenic strains of *F. oxysporum* as possible biocontrol agents of pathogenic *F. oxysporum* strains.

Trichoderma atroviride P. Karst infection and tissue colonization also results in a decrease in the disease load caused by pathogenic fungi (LU *et al.* 2004). Lu *et al.* (2004) designed an experiment in which biocontrol strains of *T. atroviride* were tagged with GFP to investigate their interaction with a number of pathogenic fungi in the plant. The authors were able to observe branching of *T. atroviride* hyphae in response to a pathogen encounter, adherence of *T. atroviride* spores to *Pythium ultimum*, and breaking points in *Rhizoctonia solani* mycelia (LU *et al.* 2004).

Conclusion

Phytophthora cinnamomi is hemibiotrophic, wide host range oomycete which has a major negative impact on agriculture, forestry and natural ecosystems. In South Africa, *P. cinnamomi* is of particular importance to the avocado industry, as it is the causal agent of PRR, which leads to significant losses in this export crop annually. PRR in avocado orchards is controlled through a combination of avocado rootstocks tolerant to *P. cinnamomi* infection, the application of chemicals and responsible farming practices. The mechanism of tolerance in rootstocks is unknown, and research aimed at unravelling the interaction between *P. cinnamomi* and avocado will aid in identification of resistance mechanisms, leading to more efficient breeding programmes. The amount of variation in pathogen populations have an effect on the durability of resistance, highlighting the importance of selecting isolates in trials. Characterization of the amount of variation in *P. cinnamomi* populations is an important component of research aimed at producing resistant rootstocks and improved control measures. A reliable transformation protocol for *P. cinnamomi* will allow for functional validation of pathogenicity genes that have been identified in *P. cinnamomi*, enabling the utilization of avirulence factors in crop protection.

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Tables

Table 1. Advantages and disadvantages associated with different techniques used for transformation of oomycetes and fungi.

Method	Principle	Advantage	Disadvantage
PMT	Preparation of protoplasts using cell wall degrading enzymes Uptake of DNA is achieved by the addition of PEG and $CaCl_2$	Different cell types (spores, germlings, hyphal tissue) can be used	Particular batch of lytic enzyme alters TR Requires regeneration procedure Copy number of DNA insertions is often high
AMT	*A. tumefaciens* carries two vectors (the binary vector containing the DNA of interest between the 24 bp border repeat and the T-vector containing the virulence region important for DNA transfer) DNA transfer is achieved during co-cultivation of *A. tumefaciens* with the fungus	Different cell types (spores, germlings, hyphal tissue) can be used Copy number of DNA insertions is low Improves targeted integration	Various parameters during co-cultivation affect TR More time-consuming than the other methods
EP	Reversible membrane permeabilisation induced by local application of electric pulses mediates DNA uptake	Different cell types (spores, germlings, hyphal tissue) can be used Simple and cheap method	Often requires protoplast formation to render cells competent
BT	Particles (tungsten, gold) are coated with DNA and become accelerated at high velocity into cells	Recipient cells can retain their cell walls (no pre-treatment required)	Requires special equipment

PMT: protoplast mediated transformation, AMT: *Agrobacterium tumefaciens*-mediated transformation; EP: electroporation; BT: biolistics transformation; TR: transformation rate. Table obtained from Meyer (2008).

Table 2. *Phytophthora* spp. transformed with fluorescent protein genes and applications of fluorescently tagged strains.

Species	Host	Method	Gene	Application	Reference
P. parasitica var *nicotiaae*	Tobacco	PEG and $CaCl_2$ mediated protoplast transformation	*GFP*	*In vitro* detection of fluorescence in hyphae, sporangia, zoospores and during zoospore encystment and germination Monitor infection process *in planta*	Bottin and Larche (1999)
P. parasitica	Tomato	PEG and $CaCl_2$ mediated protoplast transformation	*GFP*	Monitor disease progression Detection of pathogen *in planta* before the appearance of obvious external symptoms Used by Keldani *et al.* (2010) to illustrate that *P. parasitica* infects roots through appressoria	Le Berre *et al*. (2007)
P. palmivora	Cocoa	PEG and $CaCl_2$ mediated protoplast transformation	*GFP*	*In vitro* detection of fluorescence in different developmental stages	Van West *et al.* (1999)
P. sojae	Soya bean	PEG and $CaCl_2$ mediated protoplast transformation	*GFP*	*In vitro* and *in planta* detection of different developmental stages Germ tubes longer and thinner on resistant plant material in comparison to what is observed on susceptible plant material	Chen and Cheng (2009)
P. infestans	Potato and tomato	PEG and $CaCl_2$ mediated protoplast transformation	*GFP*	Monitored GFP fluorescence through life cycle Quantified induced resistance by quantifying fluorescence	Si-Ammour *et al.* (2003)
P. infestans	Potato and tomato	PEG and $CaCl_2$ mediated protoplast transformation	*GFP*	Determine success of transformation by analyzing fluorescence Fusions with markers for subcellular localization	Ah-Fong and Judelson (2011)

				Co-localization studies	
P. infestans	Potato and tomato	PEG and $CaCl_2$ mediated protoplast transformation	*GFP*	Splash dispersal of spores	Saint-Jean *et al.* (2005)
P. brassicae	Arabidopsis	PEG and $CaCl_2$ mediated protoplast transformation	*GFP*	Monitored GFP fluorescence through life cycle Quantified induced resistance by quantifying fluorescence	Si-Ammour *et al.* (2003)
P. ramorum	>80 genera of tree species	PEG and $CaCl_2$ mediated protoplast transformation	*GFP*	Monitored GFP fluorescence through life cycle/ different developmental stages	Riedel *et al.* (2009)
P. capsici	Pepper	PEG and $CaCl_2$ mediated protoplast	*GFP*, tdTomato	Monitored GFP fluorescence through life cycle/ different developmental stages Monitor disease progression Determine success of transformation by analyzing fluorescence	Dunn *et al*. (2013)

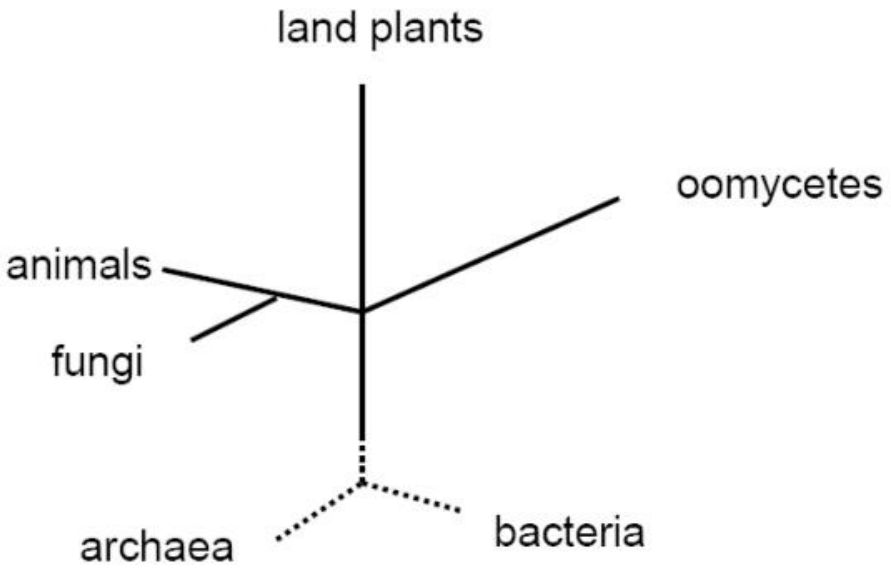

Figure 1. Cladogram depicting separation of fungi and oomycetes into distinct phylogenetic groups. Image obtained from Fry and Grünwald (2010).

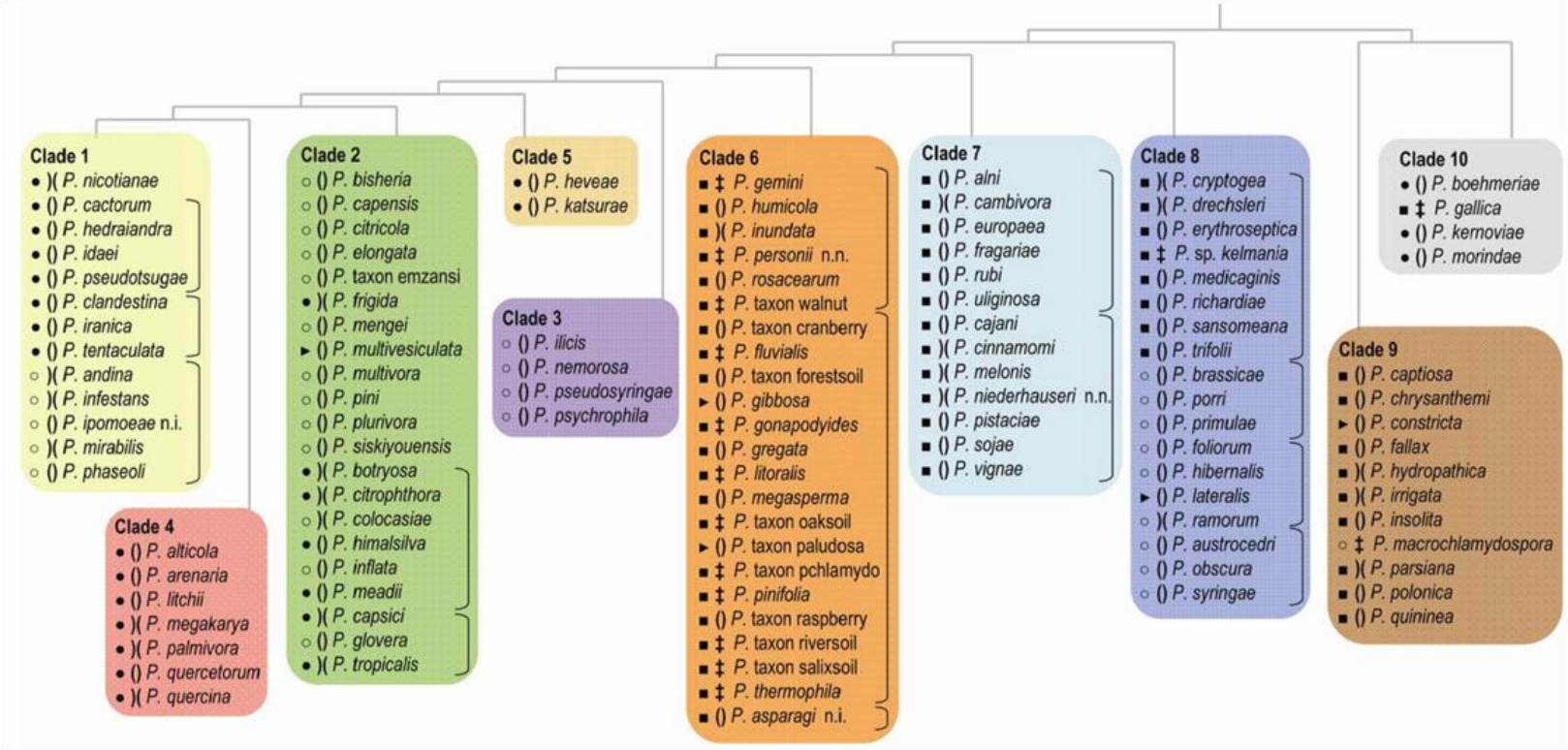

Figure 2. Diagram depicting the ten clades currently recognized within the genus *Phytophthora*. Sporangia: papillate (●), semi-papillate (○), nonpapillate (■), non- to semipapillate (►), a mixture of nonpapillate and semipapillate (►). Heterothallic species are marked as)(, homothallic species as (), and sterile species (i.e., oogonia unknown or rarely produced) as ‡. n.i. = nomen invalidum; n.n. = nomen nudum. Image obtained from Kroon *et al*. (2011).

Figure 3. Disease symptoms of *Phytophthora cinnamomi* infection on avocado. A: Die-back and a general decline in tree health; B: stem cankers and C: root rot. Images obtained online at http://www.avocadosource.com/slides/20040511/007092s.htm (A), http://www.californiaavocadogrowers.com/cultural-management-library/avocado-trunk-canker-disease-symptoms (B) and http://ucanr.edu/blogs/blogcore/postdetail.cfm?postnum= 4442 (C).

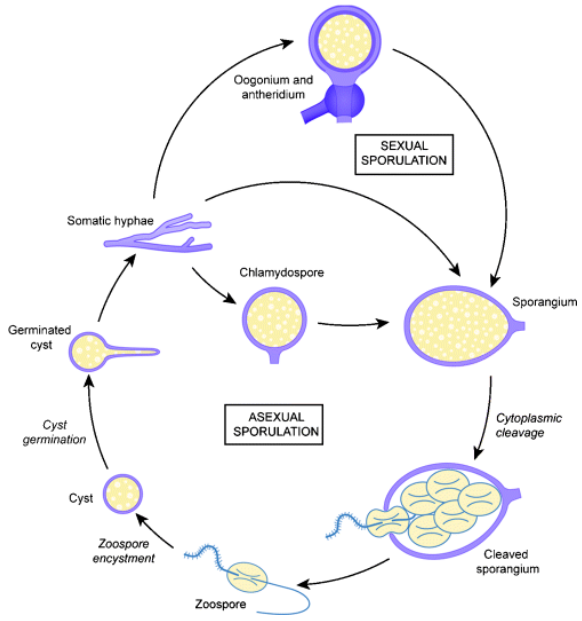

Figure 4. The life cycle of *Phytophthora cinnamomi.* Sporangia are formed from both sexual (oospores) and asexual structures (chlamydosores or hyphae). Sporangia can germinate directly through the formation of a germ tube, or can differentiate to form biflagellate zoospores. Zoospores target host tissues, attach, encyst and germinate to form hyphae. Gametes are the only haploid stage in the life cycle. Image obtained from Hardham (2005).

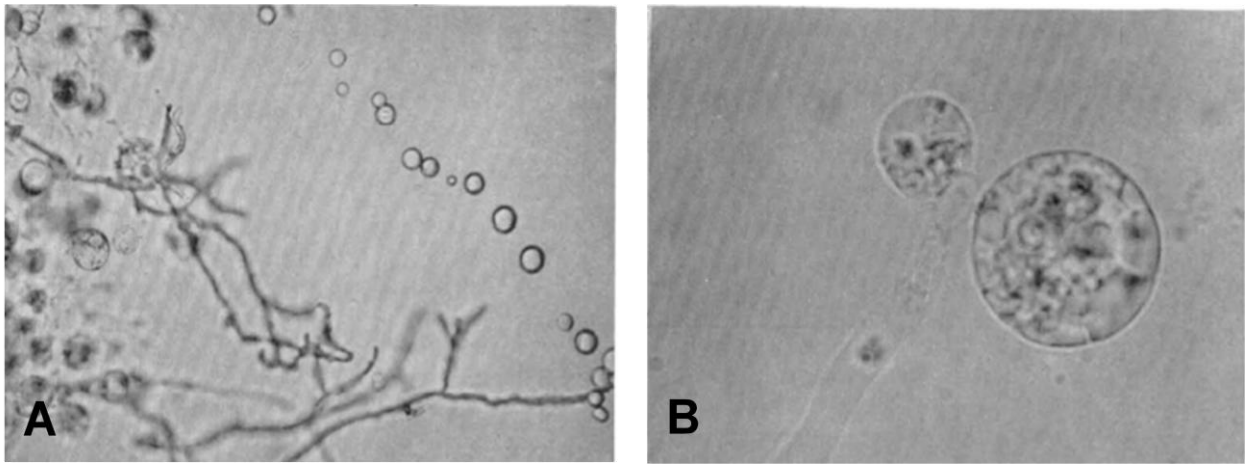

Figure 5. Protoplasts released from mycelia of *Phytophthora cinnamomi* using *Streptomyces* enzyme. A: Protoplasts formed by intercalary swelling; B: Two protoplasts released by budding through a pore in the membrane. Figure obtained from Bartnicki-Garcia and Lippman 196

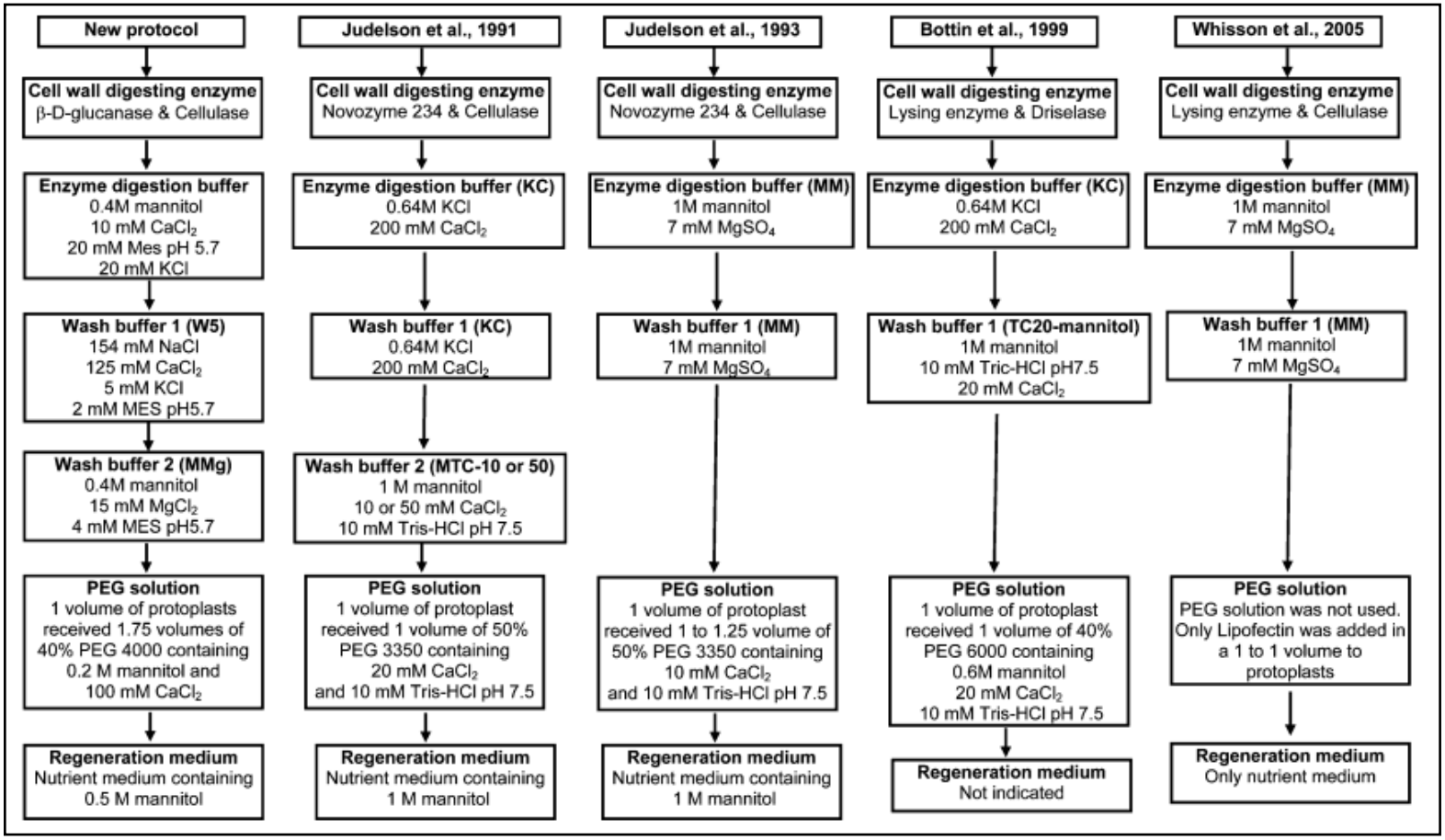

Figure 6. Comparison between the modified *Arabidopsis*-based protocol for protoplast production and previously published methods used by Judelson (1991 and 1993), Bottin (1999) and Whisson (2005) for release of protoplasts of *Phytophthora* spp. Image obtained from McLeod *et al*. (2008).

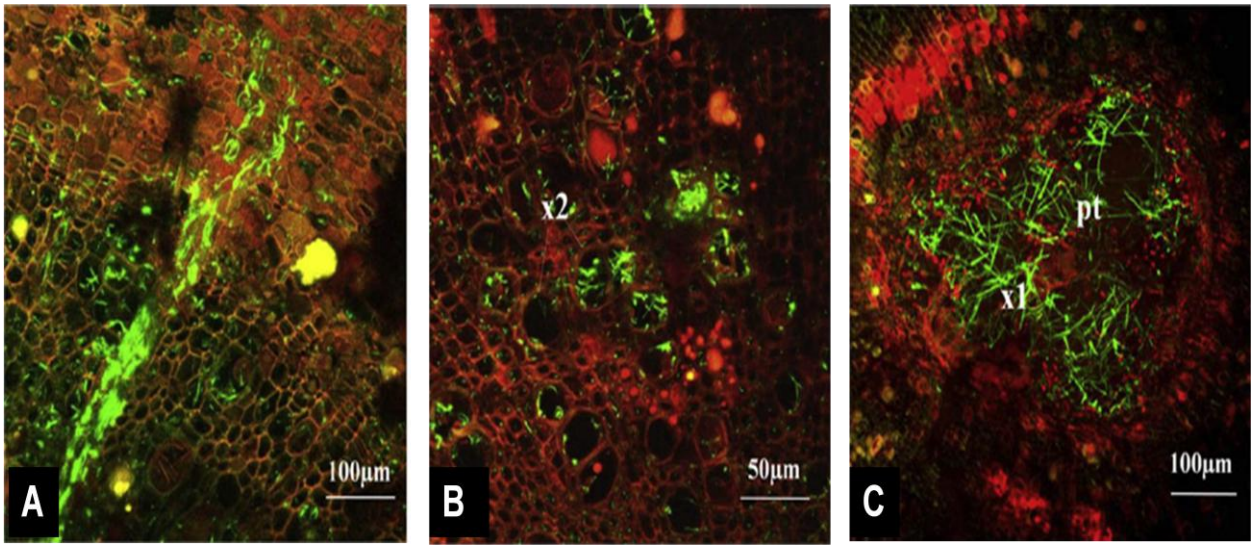

Figure 7. Visualization of *Rosellinia necatrix* infection in avocado roots using a GFP-tagged strain of *R. necatrix* and confocal laser scanning microscopy (CLSM). A and B: *R. necatrix* in the secondary xylem; C: *R. necatrix* mycelia invading the pith. Image obtained from Pliego *et al*. (2009).

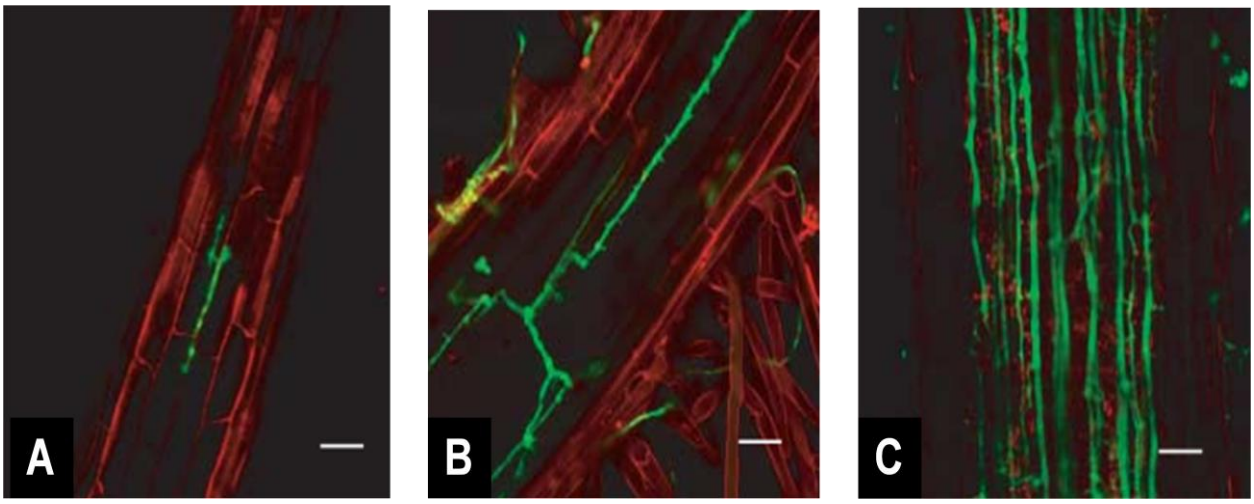

Figure 8. Detection of *Phytophthora parasitica* infection in tomato roots before the onset of external disease symptoms using a GFP-tagged strain of *Phytophthora parasitica* and confocal laser scanning microscopy (CLSM). A: Mycelia present in roots one day post-infection; B: mycelial growth in root two days post-infection, propidium iodide staining revealed that cell membranes were already destroyed at this point, and the pathogen entered its necrotrophic phase of infection; C: colonization of roots four days post-infection when first visible external disease symptoms appeared. Image obtained from Le Berre *et al.* (2009).

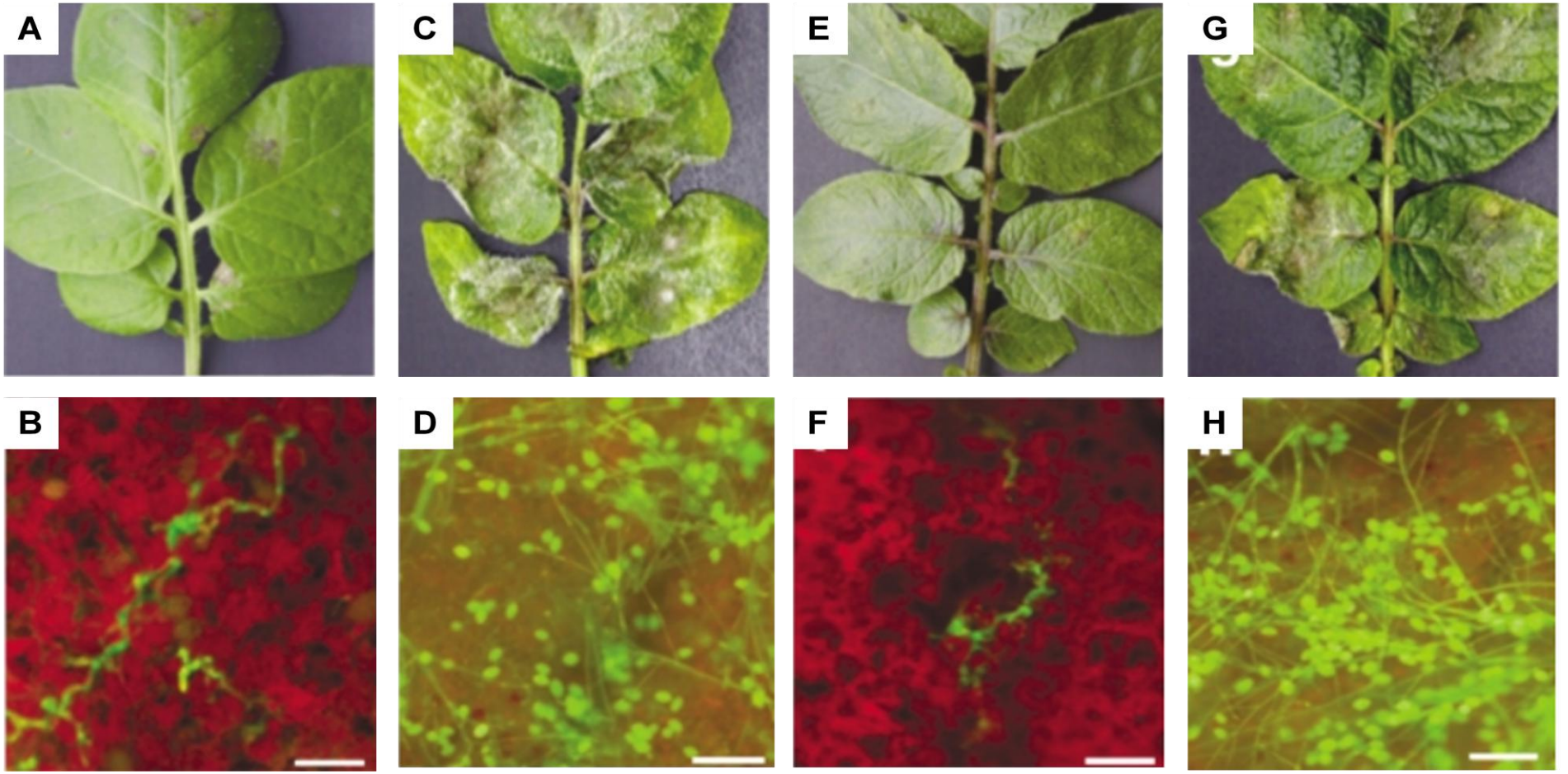

Figure 9. Quantification of induced resistance in potato against *Phytophthora infestans* at 6 dpi. A and B: Resistant cultivar; C and D: susceptible cultivar; E and F: susceptible cultivar pre-treated with β-aminobutyric acid (BABA) prior to infection; G and H: susceptible cultivar pre-treated with benzothiadiazole (BTH) prior to infection. Image obtained from Si-Ammour *et al*. 2003.

Chapter 2

Optimization of protoplast production and transformation of *Phytophthora cinnamomi*

Abstract

Fluorescently tagged pathogens are useful tools to study the development of a pathogen and its interaction with its host *in planta*. Tagging a strain of *Phytophthora cinnamomi* Rands, the causal agent of Phytophthora root rot (PRR) in *Persea americana* Mill. (avocado) with a fluorescent protein can aid in unravelling the complex host-pathogen interaction between *P. cinnamomi* and avocado. This can contribute towards identification of the mechanisms underlying tolerance in avocado rootstocks, and will aid in the development of improved control strategies for this devastating pathogen. The aim of this study was to optimize protoplast production and transformation of *P. cinnamomi* in order to produce a fluorescently tagged strain of *P. cinnamomi*. A number of published protocols used for protoplast production and transformation of *Phytophthora* spp. such as *Phytophthora infestans*, *Phytophthora sojae*, *Phytophthora citricola* and *Phytophthora aphanidermatum* were compared. Protoplasts were successfully released from *P. cinnamomi* mycelia and young germlings using three published protocols, and a PEG/$CaCl_2$ and Lipofectin-based protocol was used to transform protoplasts with plasmid DNA containing the *GFP* gene. Protoplast regeneration rates were low despite investigations into optimal mannitol concentrations in regeneration media. Two putative *P. cinnamomi* transformants were identified based on growth on selectable media and presence of the *GFP* gene, but no fluorescence was observed. Protoplast transformation of *P. cinnamomi* is hindered by relatively low protoplast regeneration and transformation efficiencies, and an *Agrobacterium*-mediated or biolistics approach could be considered for transformation of *P. cinnamomi* in future if protoplast yields and regeneration rates cannot be improved.

Introduction

The lack of knowledge on the mechanisms involved in resistance of *Persea americana* Mill. to *Phytophthora cinnamomi* Rands complicates avocado breeding and selection trials as rootstocks cannot be bred or screened for specific characteristics or markers associated with resistance. Besides the use of tolerant rootstocks, chemical control through the use of phosphite is the only other effective control strategy against PRR in avocado (AKINSANMI and DRENTH 2013; DARVAS *et al.* 1984; ESHRAGHI *et al.* 2011). However, the evolution of resistance to chemical control agents such as phosphite trunk injections is a threat (DOBROWOLSKI *et al.* 2008), and thus breeding rootstocks that are highly tolerant or resistant to *P. cinnamomi* infection should be the focus for sustainable avocado production in the future. The identification of resistance mechanisms in tolerant rootstocks requires an improved understanding of the interaction between *P. cinnamomi* and its host, avocado.

Fluorescently tagged plant pathogens are useful tools in studies aimed at unravelling plant-pathogen interactions. Applications of fluorescently tagged pathogens include quantitative *in planta* detection of pathogens (SI-AMMOUR *et al.* 2003), the ability to detect pathogens in parts of the plant where their abundance is low, pathogen detection before the onset of visible disease symptoms (LE BERRE *et al.* 2008a), the study of plant-pathogen interactions (BOTTIN *et al.* 1999; DUNN *et al.* 2013; NONOMURA *et al.* 2001; PLIEGO *et al.* 2009), tracking of genetically modified strains (BOTTIN *et al.* 1999) and *in planta* evaluation of possible biocontrol agents (LU *et al.* 2004). Induced resistance in plants to pathogens by the application of chemicals such as salicylic acid can also be monitored by quantifying fluorescence *in planta* (SI-AMMOUR *et al.* 2003).

Apart from its use in visualizing plant-pathogen interactions, the establishment of a successful, reliable and reproducible transformation protocol for *P. cinnamomi* will also be useful in other studies aimed at understanding this very important pathogen. Gene silencing by transformation with antisense RNA can be used to study pathogen avirulence factors, and will allow for functional studies in *P. cinnamomi* similar to those routinely undertaken in other *Phytophthora* spp. such as *Phytophthora infestans* (AH FONG and JUDELSON 2003; KAMOUN *et al.* 1998) and *Phytophthora sojae* (DOU *et al.* 2008b). Antisense transgenic strains of *P. cinnamomi* generated by protoplast transformation, in which elicitin genes were silenced, allowed researchers to show that silencing of the *β-cin* elicitin gene negatively affected the expression of other elicitin genes in the cluster (HORTA *et al.* 2008). A stable, fluorescently-tagged strain of *P. cinnamomi,* together with a reliable transformation protocol will be useful for future research on this important pathogen.

Four different methods are commonly used for transformation of oomycetes. Polyethyeleneglycol (PEG)-mediated protoplast transformation involves the use of cellulases and β-glucanases to remove mycelial cell walls, releasing protoplasts capable of taking up exogenous DNA. Protoplasts are osmotically stabilized in a hypertonic solution, followed by the uptake of DNA by competent protoplasts in the presence of PEG and $CaCl_2$ (BARTNICKI-GARCIA and LIPPMAN 1966; RADLEDGE and KRISTIANSEN 2001; RUIZ-DÍEZ 2002; SIETSMA *et al.* 1969). More than 11 studies have been published in which *Phytophthora* spp. were successfully transformed with fluorescent protein genes using this method (BOTTIN *et al.* 1999; CHEN *et al.* 2009; DUNN *et al.* 2013; LE BERRE *et al.* 2008a; RIEDEL *et al.* 2009; SI-AMMOUR *et al.* 2003; VAN WEST *et al.* 1999b). A large number of studies in which protoplast transformation was used to investigate gene function have been published for many species such as *P. infestans* (BLANCO and JUDELSON 2005; WHISSON *et al.* 2005), *P. sojae* (DOU *et al.* 2008a; WANG *et al.* 2009) and *P. nicotianae* (NARAYAN *et al.* 2010).

Zoospore electroporation utilizes electric shocks to render zoospore membranes more permeable, allowing for the uptake of exogenous DNA into zoospores (RUIZ-DÍEZ 2002). The major drawback of this method is the high concentration of zoospores required. *In vitro* zoospore production (especially using sterile methods) is very laborious and inconsistent in *P. cinnamomi* (CHEN and ZENTMYER 1970). Until a method capable of reliably producing high numbers of zoospores is available, transformation by zoospore electroporation of *P. cinnamomi* is improbable. Transformation through electroporation has been mostly applied in *Phytophthora infestans* (AH-FONG *et al.* 2008; BLANCO and JUDELSON 2005; LATIJNHOUWERS and GOVERS 2003) and *Phytophthora capsici* (HUITEMA *et al.* 2011). For example, in *P. infestans*, transformation by electroporation has been used to study genes involved in zoospore motility (BLANCO and JUDELSON 2005), and G-proteins which function in cell responses and development (LATIJNHOUWERS and GOVERS 2003).

Another method used for transformation of *P. cinnamomi* is microprojectile bombardment (BAILEY *et al.* 1993). The advantages of a biolistics approach is that any tissue type or structure (eg mycelia, zoospores and germinating cysts) can be transformed, which is very useful in species such as *P. cinnamomi* which sporulate poorly. This technique is also associated with a high throughput, as more than 20 shots can be performed per hour. Microprojectile bombardment frequently results in heterokaryons, but this can be easily overcome by the production of single zoospore derivatives of putative transformants (CVITANICH and JUDELSON 2003). Shortly after Judelson *et al.* (1991) transformed *P. infestans* by protoplast transformation, Bailey and co-workers reported transformation of *P. capsici*, *Phytophthora citricola*, *P. cinnamomi* and *Phytophthora citrophthora* using

microprojectile bombardment. Intact mycelia was transformed with vectors containing the *B-glucuronidase* (*GUS*) gene, and *hygromycin phosphotransferase* (*hpt*) as selectable marker (BAILEY *et al.* 1993).

Agrobacterium-mediated transformation, which is widely used for the transformation of plants, can also be used to transform fungi, yeasts and oomycetes. Transformation using *Agrobacterium tumefaciens* eliminates the need for cell wall manipulations, as with the previous two examples, proving useful when one wants to circumvent protoplast production. However, *Agrobacterium*-mediated transformation is time consuming, with a single experiment requiring approximately 17 days. *Phytophthora infestans, Phytophthora palmivora* and *Pythium ultimum* have been transformed with binary T-DNA vectors containing the *GUS* and *neomycin phosphotransferase* (*npt)* genes using *Agrobacterium*-mediated zoospore transformation (VIJN and GOVERS 2003). Following co-cultivation of *Agrobacterium* with germinating cysts, young germlings were transferred to a selectable agar medium (geneticin) for identification of putative transformants. Up to 30 transformants were obtained per $1x10^7$ zoospores, with most transformants containing a single copy of the T-DNA incorporated at a random chromosomal locus (VIJN and GOVERS 2003).

Despite the low and inconsistent rates of obtaining stable transformants using a protoplast and PEG/$CaCl_2$ approach, this method remains the most successful, and was selected for transformation of *P. cinnamomi* in this study. The first step in the establishment of a successful and reliable protoplast transformation protocol is to obtain large numbers of high quality protoplasts, and an ability to regenerate protoplasts after transformation (AH-FONG and JUDELSON 2011; JUDELSON *et al.* 1991; MCLEOD *et al.* 2008). The first aim of this study was to evaluate two previously published protocols used to transform *P. infestans* (JUDELSON *et al*. 1991; MCLEOD *et al.* 2008), *P. cinnamomi* (HORTA *et al.* 2008; JUDELSON *et al.* 1991), *P. sojae*, *P. citricola* and *Phytophthora aphanidermatum* (MCLEOD *et al.* 2008) for releasing protoplasts from *P. cinnamomi*. The second aim was to determine the optimal mannitol concentration for regeneration of protoplasts, and the third aim involved combining results of the first two aims in order to transform *P. cinnamomi* with a fluorescent protein marker gene.

Materials and Methods

Isolates

Five isolates, 941, 942, 943, 2060 and TBB5T7S3 were used in this study **(Table 1)**. Isolates 941, 942, 943 and 2060 were provided by Dr Akif Eskalen from the Department of Microbiology and Plant Pathology at the University of California, Riverside. Isolates 941, 942, 943 and 2060 were isolated from diseased avocado trees (trunks and roots) in avocado orchards in California, USA, and TBB5T7S3 was baited from a soil sample collected in close proximity to diseased avocado roots in an avocado orchard in Tzaneen, Limpopo province, South Africa. Isolates were routinely cultured on 5% V8 agar (50 ml cleared V8 juice (Campbell Soup Company, USA), 0.5 g $CaCO_3$, 18 g agar (Difco, BD Diagnostic Systems, Maryland, USA) and dH_2O to 1000 ml, 50 µg/ml ampicillin (Sigma-Aldrich, Taufkirchen, Germany), 4 ml/l nystatin (Sigma-Aldrich)) at 25°C in the dark. All isolates are now maintained in the culture collection of the Fruit Tree Biotechnology Programme at the University of Pretoria, South Africa.

DNA extractions

Cultures were grown on 20% V8 agar (200 ml cleared V8 juice (Campbell Soup Company), 2 g $CaCO_3$, 18 g agar (Difco) and dH_2O to 1000 ml, with 50 µg/ml ampicillin (Sigma-Aldrich), 4 ml/l nystatin (Sigma-Aldrich)) for 1 week to generate aerial hyphae for DNA extraction. DNA was extracted using PrepMan Ultra Reagent™ (Applied Biosystems, Foster City, California, USA). Aerial mycelia from two 90 mm agar plates were incubated with 50 µl of Prepman reagent at 95°C for 10 min. Mycelia were homogenized with a 1ml pipette tip, and incubated for 5 min at 95°C. Supernatant was recovered by centrifugation at 10 000 rpm for 10 min using a Centrifuge 5810 R (Eppendorf, Hamburg, Germany). Concentrations and purity of extracted DNA was measured using a NanoDrop ND-1000 spectrophotometer and NanoDrop 3.2.1 Software (Nanodrop Technologies, Inc., Montchanin, Delaware, USA). DNA was analyzed by agarose gel electrophoresis (2%, 80 V), and visualized under UV light through the addition of GelRed (Biotium Inc., California, USA).

Identification of isolates as *P. cinnamomi* using molecular techniques

Polymerase chain reaction (PCR) amplification of the species-specific LPV3 fragment was used to confirm the identity of the five isolates as *P. cinnamomi* (KONG *et al.* 2003). Each

LPV3 PCR reaction (20 µl) contained 20-50 ng of template DNA, 2 µl 10x Fast Start buffer (Roche Applied Science, Mannheim, Germany), 200 µM of each dNTP, 0.2 µM LPV3 sense primer (5' GTGCAGACTGTCGATGTG 3'), 0.2 µM LPV3 antisense primer (5' GAACCACAACAGGCACGT 3') and 1 unit Fast Start Taq DNA Polymerase (5 units/µl) (Roche). The PCR was carried out on a 2720 Thermal Cycler (Applied Biosystems, Singapore), and the PCR program consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, and a final extension step at 72°C for 10 min. PCR products were visualized under UV light using GelRed (Biotium Inc.) following agarose gel electrophoresis (1%, 80V).

Comparison of sporulation ability of US *P. cinnamomi* isolates

A protocol published by Chen and Zentmyer (1970) was used to obtain sporulating mycelia in sterile culture. Five percent V8 (50 ml cleared V8 juice, 0.5 g $CaCO_3$, 20 mg/1000 ml β-sitosterol (ICN Biomedicals, Inc., Ohio, USA), 50 µg/ml ampicillin (Sigma-Aldrich), 4 ml/l nystatin (Sigma-Aldrich) and dH_2O to 1000 ml) and pea broth (supernatant of 200 g frozen peas blended in 500 ml dH_2O, with dH_2O up to 1000 ml, 50 µg/ml ampicillin (Sigma-Aldrich), 4 ml/l nystatin (Sigma-Aldrich)) were inoculated with three day old cultures of each of the four isolates growing on 10% V8 agar (100 ml cleared V8 juice, 1 g $CaCO_3$ and dH_2O to 1000 ml, 50 µg/ml ampicillin (Sigma-Aldrich), 4 ml/l nystatin (Sigma-Aldrich)). After two days incubation at room temperature under fluorescent lights, plates were washed four times with a mineral salts solution (CHEN and ZENTMYER 1970), and incubated for another two days at room temperature under fluorescent lights. After the second two day incubation period, four plates per isolate and broth medium were examined for the presence of sporangia using a microscope. The highest sporulating isolate was selected for subsequent experiments.

Plasmid extraction, purification and design and testing of transgene specific primers

Plasmid pGFPH **(Figure 1)** was provided by Prof Howard Judelson (University of California, Riverside, USA). Plasmids were extracted from *E. coli* strain JM109 cells using a Pure Yield™ Plasmid Maxiprep system (Promega, Wisconsin, USA) according to the manufacturer's instructions. Plasmid DNA concentration and quality were determined as described. PCR primers able to amplify fragments of the *GFP* and *hpt* genes were designed using Primer Premier 5.0 software (Premier Biosoft, California, USA). The sequences of the two respective genes are available online (http://oomyceteworld.net/plasmids/plasmids.html).

Oligonucleotides were synthesized by Integrated DNA Technologies (IDT) (Coralville, USA), and primer specificity was analyzed by conventional PCR. *GFP* and *hpt* genes were amplified from pGFPH plasmid DNA. Each 20 µl PCR reaction consisted of 2 µl of template plasmid DNA (1:1000 dilution of plasmid DNA), 2 µl of 10x Fast Start buffer (Roche Applied Science, Mannheim, Germany), 200 µM of each dNTP, 0.2 µM of the sense primer, 0.2 µM of the antisense primer and 1 unit of Fast Start Taq DNA Polymerase (5 units/µl) (Roche). The PCR program consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, and a final extension step at 72°C for 10 min in a 2720 Thermal Cycler (Applied Biosystems). Plasmid DNA and PCR products were analyzed by agarose gel electrophoresis (1%, 80 V).

Antibiotic growth trial to determine minimum hygromycin B concentration capable of inhibiting *P. cinnamomi* growth

Isolate 943 was transferred onto 90 mm petri dishes containing 20 ml of 5% V8 agar amended with different amounts of the antibiotic hygromycin B (Sigma-Aldrich) to determine the minimum concentration of the antibiotic able to restrict growth of *P. cinnamomi*. This was required to establish the concentration of hygromycin B to be used in selection plates during transformation experiments. Hygromycin B concentrations evaluated included 20 µg/ml, 50 µg/ml, 80 µg/ml, 100 µg/ml and 0 µg/ml (control). Plates were inspected for the presence of growth after one week's incubation at 25°C in the dark.

Release of protoplasts from one-day-old *P. cinnamomi* cultures

Cultures were initiated by inoculating 5% V8 broth with three-day-old *P. cinnamomi* cultures through a syringe, followed by incubation for one day in the dark at room temperature. Protoplasts were released from mycelia using two different protocols published by Judelson *et al.* (1991) and McLeod *et al*. (2008). A modified version of the protocol published by Judelson *et al*. (1991) involved washing mycelia in 40 ml Fry Protoplasting Buffer (FPB) (0.4 M mannitol, 20 mM KCl, 20 mM MES pH 5.7, 10 mM $CaCl_2$) before digestion (available online at http://oomyceteworld.net/protocols/Protoplast%20transformation.pdf). Mycelia were digested by adding 3 ml of enzyme digestion solution (5 mg/ml cellulase from *Trichoderma reesi* (Sigma-Aldrich), 10 mg/ml β-glucanase (Extralyse, Laffort, South Africa) dissolved in FPB to 1 ml of mycelia. Mycelia was incubated in enzyme digestion solution with gentle shaking (40 rpm on an orbital platform) at room temperature for 40 min. Protoplasts were

separated from mycelial debris by passing the protoplast mixture through a 50 µm nylon membrane (Biodesign, New York, USA), and protoplasts harvested by centrifugation using a swinging bucket rotor (Centrifuge 5810 R, Eppendorf) (to ensure that protoplasts pellet at the bottom of the tube) at 700 x g for 4 min at room temperature. Pelleted protoplasts were washed three times in 30 ml FPB, 15 ml FPB + 15 ml MT solution (1 M mannitol, 10 mM Tris pH 5.7) and 30 ml MT + $CaCl_2$ (1 M mannitol, 10 mM Tris pH 5.7, 10 mM $CaCl_2$) respectively by resuspending pelleted protoplasts in wash buffers followed by centrifugation at 700 x g for 4 min at room temperature. Protoplasts were resuspended in 1 ml MT + $CaCl_2$, and protoplast concentrations determined using a haemocytometer (Marienfeld Laboratory Glassware, Lauda-Königshofen, Germany). Concentrations were averaged from two biological replicates. The second protocol published by McLeod *et al*. (2008) required that mycelial mats were rinsed twice with 1 M mannitol, followed by a 10 min incubation in 1 M mannitol prior to protoplast release. Mycelial mats were digested in 14 vol of enzyme digestion solution (1% (w/v) β-glucanase (Extralyse), 7 µl/ml cellulose from *T. reesi* (Sigma-Aldrich), 0.4 M mannitol, 20 mM KCl, 20 mM MES pH 5.7, 10 mM $CaCl_2$) to 1 ml mycelia for 1 hr with gentle shaking (40 rpm). Protoplasts were harvested by passing the enzyme digestion solution through a 50 µm nylon membrane (Biodesign), followed by centrifugation at 750 x g for 3 min using a swinging bucket rotor. Protoplasts were washed in 25 ml W5 buffer (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES pH 5.7) by centrifugation at 750 x g for 3 min, followed by a 30 min incubation on ice in 5 ml W5 buffer. Protoplasts were collected by centrifugation at 750 x g for 3 min, and resuspended in 1 ml Mmg (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES pH5.7) solution. Protoplasts were photographed with a AxioCam MRc (Zeiss, Göttingen, Germany) coupled to the Axioskop microscope (Zeiss).

Release of protoplasts from two-day-old *P. cinnamomi* cultures and young germlings

Three-day-old cultures were used to inoculate 5% V8 broth using a syringe, followed by incubation for two days at room temperature. Protoplasts were released from mycelia using two different methods as described above. Sporulating mycelia and zoospores were produced as previously described, and zoospores were incubated in 5% V8 broth for 24 hr at room temperature in the dark to allow for germination of zoospores and the formation of thin, uniform mycelial mats. Protoplasts were released from young germlings using the modified protocol of Judelson *et al*. (1991) and a method described by Horta and co-workers (2008) for the transformation of *P. cinnamomi*. The method used by Horta was based on the protocol for protoplast production and transformation in *P. infestans* described by Judelson, with a few modifications. Enzyme digestion solution (0.64 M KCl, 0.2 M $CaCl_2$) contained 7

mg/ml of both β-glucanase and cellulose, and mycelia were digested for 2.5 hr at 25 °C. Stereomicroscope images of protoplasts were obtained as described above.

Evaluation of optimal mannitol concentration for regeneration of protoplasts

One- and two-day-old cultures were generated for digestion as described above. Protoplasts were released from *P. cinnamomi* cultures using the modified version of the method described by Judelson *et al*. (1991). Protoplasts were harvested by centrifugation at 700 x g for 4 min (Centrifuge 5810 R, Eppendorf), and regenerated overnight at room temperature in 5% V8 broth containing 0.5, 0.6, 0.7, 0.8, and 0.9 M mannitol in order to establish the optimal mannitol concentration for regeneration of *P. cinnamomi* protoplasts. Regenerated protoplasts were collected by centrifugation, and the number of protoplasts with germtubes was determined using a haemocytometer, and percentage regeneration calculated. Images of regenerating protoplasts were obtained as previously described.

PEG and Lipofectin mediated transformation of *P. cinnamomi* protoplasts

A thin mycelial mat of young germlings was produced from isolate 943 as previously described. Protoplasts were released and transformed according to the modified version of the Judelson method, with the use of Lipofectin (Invitrogen Life Sciences, California, USA). Approximately 1.5 ml of germlings were digested in 8 ml of enzyme digestion solution for 40 min with gentle shaking (40 rpm on an orbital platform), and protoplasts were transformed with 20 µg pGFPH DNA complexed wih 60 µl Lipofectin. Protoplasts were released from TBB5T7S3 by digesting mycelia in 35 ml of enzyme digestion solution containing 400 µl cellulase (Sigma) and 400 µg lysing enzyme (Sigma), according to the method described by McLeod *et al.* (2008). Protoplasts were transformed with 40 µg pGFPH plasmid DNA complexed with 60 µl Lipofectin. Protoplasts were regenerated for 24 hr in 5% V8 broth containing 0.6 M of mannitol at room temperature. After 24 hours, protoplasts were examined under a microscope using a haemocytometer to determine whether regeneration occurred, and plated onto 5% V8 agar with 80 µg/ml hygromycin B following harvesting by centrifugation. Regenerating protoplasts were photographed.

Confirmation of presence of transgenes in putative *P. cinnamomi* transformants

Colonies on transformation selection plates containing hygromycin B were transferred to fresh 5% V8 agar selection plates containing 20 µg/ml hygromycin B to maintain single colonies and to prevent contamination of putative transformants with bacteria present on original selection plates. After 7-10 days, hyphae was scraped from colonies, and used to inoculate YM broth (20 g malt extract (Biolab, Midrand, South Africa), 2 g yeast extract (Biolab), dH_2O to 1000 ml), and incubated for 2.5 days at 25°C with gentle shaking. Mycelia were harvested, and DNA extracted as previously described. *GFP* and *hpt* gene-specific PCRs were completed as described above. After colonies were allowed to grow for 14 days a DFP-1^{TM} Dual Fluorescent Protein Flashlight (NightSea, Massachussets, USA) and fluorescence microscopy (Zeiss Axiovert200 fluorescence microscope (Munchen, Germany)) was used to evaluate putative transformants for fluorescence.

Results

DNA extraction and identification of isolates using molecular techniques

Genomic DNA was successfully extracted from pure cultures of isolates 941, 942, 943, 2060 and TBB5T7S3. Isolates 941, 942, 943, 2060 and TBB5T7S3 were identified as *P. cinnamomi* using the LPV3 primer pair which successfully amplified a species-specific 450 bp fragment of the *Lpv* gene by PCR **(Figure 2)**.

Comparison of sporulation ability of *P. cinnamomi* isolates

Mycelia of pea broth cultures tended to clump together, with a cotton wool appearance, while 5% V8 broth cultures had a uniform, even appearance **(Figure 3)**. Sporangia were only present in isolates 941 and 943 grown in 5% V8 broth prior to mineral salt washing steps **(Table 2)**. In all other cultures only chlamydospores were observed. Zoospores were released from sporangia present in cultures of isolates 941 and 943 by exposure to a brief cold shock, and were harvested after 90 minutes. The zoospore yield for isolate 941 was $3x10^4$ zoospores/ml, and that of isolate 943 was $5x10^4$ zoospores/ml. Isolate 943 was used in subsequent experiments due to its higher sporulation ability.

Plasmid extraction and purification

High quality pGFPH plasmid DNA was obtained for use in transformation experiments **(Figure 4)**. The plasmid DNA concentration obtained was 939.5 ng/µl, and the A260/280 absorbance for plasmid DNA was 1.92, indicating isolation of pure DNA with little contaminating proteins and organic compounds.

Design and testing of GFP and hygromycin phosphotransferase specific primers

GFP and *hpt* gene specific primers were designed to confirm genes present in plasmids before use in transformation experiments, and for use in confirming the presence of transgenes in putative transformants **(Table 3)**. The eGFP primer pair amplified a 295 bp fragment of the *eGFP* gene, and the hpt primer pair a 484 bp fragment of the *hpt* gene **(Figure 5)**. All primer pairs successfully amplified specific regions of the respective genes, as designed, yielding PCR products of expected sizes.

Antibiotic growth trial to determine minimum hygromycin B concentration capable of inhibiting *P. cinnamomi* growth

Control plates deficient in hygromycin B showed a significant amount of growth as expected **(Table 4)**. Growth was observed on plates containing 20 µg/ml and 50 µg/ml of hygromycin B, and these concentrations are thus too low to inhibit the growth of non-transformed *P. cinnamomi*. However, both 80 µg/ml and 100 µg/ml of hygromycin B successfully inhibited the growth of *P. cinnamomi*. Consequently, selection plates in transformation experiments of isolate 943 contained 80 µg/ml of the antibiotic hygromycin B to enable selection of putative true transformants.

Release of protoplasts from one-day-old *Phytophthora cinnamomi* cultures

Following one days' growth, cultures were very small, with the agar plugs very large in comparison to the amount of mycelia, unavoidably resulting in the presence of agar plugs in the enzyme digestion solution. Using the method described by Judelson *et al.* (1991), an average of 33.5×10^4 protoplasts/ml was obtained, and an average of 15.5×10^4 protoplasts/ml for the protocol published by McLeod *et al.* (2008). Protoplasting using the Judelson protocol resulted in the release of a greater number (more than double) of protoplasts compared to

the protocol of McLeod. Despite the limited amount of starting material, the digestion was not 100% complete, with pieces of mycelia still present after digestion.

Release of protoplasts from two-day-old *P. cinnamomi* cultures

The modified version of the method described by Judelson *et al*. (1991) generated an average of $2.6x10^5$ protoplasts/ml, and the method of McLeod *et al.* (2008) yielded an average of $2.5x10^5$ protoplasts/ml. Protoplasts obtained differed in size **(Figure 6)**. As was the case in the above mentioned experiment, pieces of mycelia were still present after completion of the incubation time, indicating that the protoplast production reaction was not fully complete.

Release of protoplasts from young *P. cinnamomi* germlings

After one day incubation, young, thin mycelial mats were present at the bottom of flasks in which zoospores were germinating in 5% V8 broth **(Figure 7 A and B)**. An average of $4.5x10^6$ protoplasts/ml were obtained, an amount sufficient for a transformation experiment. Protoplasts were also more uniform in size **(Figure 7 C and D)**, compared to those obtained when digesting mycelia, which could be indicative of increased protoplast quality. Protoplasting of young germlings according to the method of Horta and co-workers (2008) resulted in average protoplast yields of $8.4x10^5$ protoplasts/ml. Despite the longer digestion time, small pieces of mycelia were still present in the enzyme digestion buffer following digestion.

Evaluation of optimal mannitol concentration for regeneration of protoplasts

The optimum concentration of mannitol in regeneration media was found to be between 0.5 and 0.8 M **(Tables 5 and 6)**. Protoplast regeneration rates increased with increasing mannitol concentrations, and reached a maximum at 0.7 M mannitol, after which the percentage regeneration declined. At lower concentrations of mannitol, such as 0.5 M, germtubes of regenerated protoplasts grew very fast, as seen in **Figures 8 A, B** and **C**, where germtubes had already started to branch after 24 hr. **Figure 8 D** shows the degree of regeneration following regeneration for 48 hr in regeneration media containing 0.5 M mannitol. Plating out protoplasts with such long germtubes on selection plates following

harvesting by centrifugation would prevent formation of single colonies on selection plates. At higher mannitol concentrations (0.8 M), germtubes were shorter, with little or no branching observed **(Figure 8 E)**, which is more desirable for obtaining single colonies. Only a small fraction of protoplasts were able to successfully regenerate their cell walls and form germtubes **(Figure 8 F)**.

PEG and Lipofectin mediated transformation of *P. cinnamomi* protoplasts

Protoplast yield after digestion and filtration was $2.7x10^7$ protoplasts/ml. Following the washing steps, the amount of protoplasts present was determined, and $8.75x10^6$ protoplasts were transformed with 40 µg of pGFPH plasmid DNA. Regeneration of protoplasts **(Figure 9 A)** and structures resembling germinating cysts with appressoria extending out of the protoplasts were observed **(Figure 9 B).** Fifty putative transformants appeared on selection plates following one week of incubation.

Confirmation of presence of transgenes in putative *P. cinnamomi* transformants

Two putative transformant colonies were identified following amplification of a 484 bp fragment of the *GFP* **(Figure 10)**. However, the presence of the *hpt* gene was not confirmed in the two colonies, despite them being able to grow on the selection medium. Following molecular confirmation of the presence of the transgene, colonies were assessed for fluorescence, but neither colony emitted fluorescence.

Discussion

In this study, protoplasts were successfully released from one- and two day old cultures and young germlings of *P. cinnamomi* using three previously described protoplast production and transformation protocols. The optimal mannitol concentration for regeneration of *P. cinnamomi* protoplasts was determined to be between 0.5 - 0.8 M, and protoplast cell walls were successfully regenerated following digestion. Following PEG and $CaCl_2$-mediated transformation of protoplasts released from TBB5T7S3, two putative GFP transformants were identified based on the presence of the *GFP* gene. Despite the presence of the GFP gene, no colonies were able to fluoresce.

With the publication of the genome and transcriptome sequences of many *Phytophthora* spp., the establishment of reliable and efficient transformation protocols has become increasingly important in order to facilitate functional analysis of sequence data generated by these projects (MCLEOD *et al.* 2008). In spite of the large number of *Phytophthora* spp. that have been transformed to date, routine transformation of oomycetes is still troublesome and inefficient (GOVERS and GIJZEN 2006; MCLEOD *et al.* 2008). Protoplast transformation is the most successful method of transformation in oomycetes, and thus often the method of choice, but the generation of stable transformants is inconsistent, with relatively low success rates (MCLEOD *et al.* 2008). The major obstacles of protoplast transformation are the large amounts of starting material required, and the low regeneration rates of protoplasts following transformation (MCLEOD *et al.* 2008).

The aim of this study was to transform *P. cinnamomi* with the fluorescent protein gene *GFP* to serve two purposes. The first was to establish a reliable transformation protocol for *P. cinnamomi* in our laboratory, and fluorescent protein genes are useful markers when optimizing transformation protocols. Secondly, a fluorescently-tagged strain of *P. cinnamomi* would also be useful for studying the interaction between *P. cinnamomi* and its host, avocado *in planta,* which might aid in the identification of resistance mechanisms in *P. americana* rootstocks. Failure to obtain transformants using the method described by Horta *et al.* in 2008 for transformation of *P. cinnamomi* protoplasts necessitated an alternative approach to obtain *P. cinnamomi* transformants. In the protocol described by Horta, which is based on the method of Judelson *et al.* (1991), protoplasts were released from three-day-old mycelia. Alternative methods for releasing protoplasts, different starting materials and mannitol concentrations were evaluated, with the hope of identifying a combination with which *P. cinnamomi* could be successfully transformed. These included protocols successfully employed in other *Phytophthora* spp., and investigation into the use of young germlings, which is frequently used as starting material for protoplast transformation in *P. infestans* (CAMPBELL *et al.* 1989).

Following successful DNA extraction and confirmation of identity of isolates as *P. cinnamomi*, the four US isolates used in this study were evaluated for their ability to produce sporulating mycelia in sterile culture using the method described by Chen and Zentmeyer (1970). The production of thin, uniform mycelial mats of young germlings requires germination of zoospores in liquid broth overnight. The production of *P. cinnamomi* zoospores is notoriously unreliable (CHEE and NEWHOOK 1966; CHEN and ZENTMYER 1970; ZENTMYER and MARSHALL 1959), especially in axenic culture, as zoospore production using river water usually results in higher zoospore yields (Anandi Reitmann, unpublished work).

Unfortunately, for certain applications such as transformation, producing zoospores using river water is not possible as contamination has to be avoided, but for others such as infection of plant material this method is most widely used (MAHOMED and VAN DEN BERG 2011; REEKSTING *et al.* 2014). Sporangia were only produced in response to nutrient and light stress in isolates 941 and 943 grown in V8 broth. Isolates grown in pea broth appeared clumpy, which could have prevented efficient removal of nutrients during washing steps, impairing sporulation. The uniform growth of *P. cinnamomi* in V8 broth thus appears to be more suitable for producing sporulating mycelia.

The inconsistent and unreliable nature of zoospore production for *P. cinnamomi* complicates studies aimed at unravelling the basic biology of this economically important pathogen (CHEN and ZENTMYER 1970). The identification of high sporulating isolates and methods that enable consistent and reliable production of larger numbers of zoospores will allow for the production of multiple mats of young germlings, enabling multiple transformation reactions simultaneously. The ability to produce large numbers of zoospores will also open up the possibility of using zoospore electroporation for transformation, which has been successfully used to transform other *Phytophthora* spp, for transformation of *P. cinnamomi* (LATIJNHOUWERS *et al*. 2004).

Typically 2-5x10^5 zoospores/ml (final concentration of zoospores in broth) is required to inoculate liquid media in order to generate young mycelial mats from which to release protoplasts. Using the method described by Chen and Zentmyer (1970) and isolate 943, only 2x10^4 zoospores/ml were obtained on average in different attempts, but mycelial mats were successfully produced with these relatively low yields of zoospores by concentrating zoospores by centrifugation in attempts where zoospore yield was very low. A minimum of 15, 150-mm petri dishes should be inoculated in order to generate enough sporulating mycelia and zoospores to form one mycelial mat in a 1 l Erlenmeyer flask. For successful formation of mycelial mats consisting of young germlings, flasks should not be filled with broth to more than 10% of their final volume, to allow for sufficient aeration.

Establishing the minimum concentration of hygromycin B capable of preventing growth of wild type *P. cinnamomi* is very important for efficient selection of putative transformants. Antibiotic concentrations which are too low will result in false positives, while excessively high concentrations will lead to false negatives. The concentration of antibiotics required for selection of transformants not only differs between species, but also among isolates of the same species. The concentration of antibiotics required in selection media should thus be determined for each individual isolate used in transformation experiments. Evaluating the

growth of isolate 943 on 5% V8 agar with increasing concentrations of hygromycin B enabled the identification of 80 µg/ml of hygromycin B as a suitable concentration for use in transformation selection plates.

In all experiments, protoplast yield was the highest when using the modified method of Judelson *et al*. (1991). Despite obtaining protoplasts, and also observing some regeneration, using one-day-old mycelia for protoplast production is not ideal. After one day's growth very little mycelia is present, resulting in a low protoplast yield. The effect of relatively high amounts of agar in the enzyme digestion mixture on digestion and protoplast stability can also not be discounted. Protoplast yield was higher, as expected, with the digestion of two-day-old cultures of *P. cinnamomi*, as more starting material was present. Differences observed in the sizes of the protoplasts can be attributed to the fact that the starting material (mycelia) was not uniform in age, and also too old, negatively affecting protoplast quality. Variation in protoplast size, internal morphology of protoplasts and time to protoplast detachment has been reported in previous protoplasting experiments of *P. cinnamomi* mycelia (BARTNICKI-GARCIA and LIPPMAN 1966). Increasing mannitol concentration might prevent the formation of large protoplasts.

Releasing protoplasts from young germlings resulted in the highest yield of protoplasts when compared to the other two experiments, and protoplasts were also more similar in size. Protoplasting of mycelia generally takes longer (in comparison to digesting young germlings), with pieces of mycelia still persisting after 40 minutes of enzyme digestion, which is rarely the case when digesting young germlings. For protoplasting, young and uniform starting material is crucial, and the presence of mycelia after digestion could be due to the lack of uniformity in the starting material. It was concluded that young germlings are the best starting material to use in order to ensure that large numbers of high quality protoplasts are obtained. The number of protoplasts obtained varied widely under identical conditions, corresponding to the inconsistency reported in literature (BARTNICKI-GARCIA and LIPPMAN 1966; GOVERS and GIJZEN 2006; MCLEOD *et al.* 2008).

The method described by McLeod *et a*l. (2008) is based on a protocol used for the release of protoplasts from *Arabidopsis* mesophyll cells (SHEEN 2002), which has been modified for oomycetes in a bid to consistently produce larger numbers of protoplasts in order to improve the frequency of stable transformation of oomycetes. One of the reasons cited by McLeod and co-workers for their attempts at finding alternative protoplasting methods was the unavailability of Novozyme 234, the enzyme used by Judelson and co-workers in the development of the first oomycete protoplast transformation protocols (JUDELSON and

MICHELMORE 1991; JUDELSON *et al.* 1991). However, the results of this and other studies prove that alternative β-glucanases are able to successfully release protoplasts from tissue of *Phytophthora* spp. (AH-FONG and JUDELSON 2011; HORTA *et al.* 2008).

Mannitol acts as an osmoticum, preventing protoplasts from lysing during digestion and cell wall regeneration, and also reduces the growth of germtubes during germination of protoplasts. At high mannitol concentrations (> 1 M), protoplasts do not regenerate, and at low mannitol concentrations (< 0.5 M) germtubes grow very fast, and intertwine easily, which might prevent recovery of single colonies on transformation selection plates. The optimum concentration of mannitol required for regeneration is a balance between regeneration rates and the length of the germtubes. In this study, regeneration of protoplasts was observed at mannitol concentrations between 0.5 M – 0.8 M, with a peak in regeneration rates at 0.7 M mannitol. In this range regeneration still occurred, but germtubes did not grow too fast. This was in the expected range, as *P. infestans*, *P. citricola*, *P. aphanidermatum, P. sojae* and *P. cinnamomi* protoplasts are generally regenerated in nutrient media containing 0.5 M mannitol (HORTA *et al.* 2008; MCLEOD *et al.* 2008), while Judelson regenerates protoplasts in nutrient media containing 1 M mannitol (JUDELSON and MICHELMORE 1991; JUDELSON *et al.* 1991). The fact that protoplasts of *P. infestans* can be regenerated at two such different concentrations (0.5 M and 1 M) suggests that within a certain range, mannitol concentration in nutrient regeneration media is not that crucial.

Approximately half of protoplasts are lost during washing steps performed prior to transformation. A minimum of 2 - $3x10^7$ protoplasts should thus be obtained after digestion to ensure approximately 1 - $1.5x10^7$ protoplasts are transformed. Despite transforming sufficient numbers of protoplasts in a number of different experiments, and observing regeneration of protoplasts, putative transformants were only obtained in one experiment. Following confirmation of the presence of the *GFP* gene colonies were allowed to grow for 14 days before evaluation of colony fluorescence.

Possible explanations for the lack of fluorescence include loss of the transgenes, integration of plasmids into sites in the genome which lead to silencing of expression or the coenocytic nature of *P. cinnamomi* hyphae. Studies in *Phytophthora parasitica* have indicated that transgenes are frequently lost from primary transformants (GAULIN *et al.* 2007). Two out of three transformants lost the *hptII* gene following sub-culturing of transformants on non-selective media, in contrast to the stable transformation reported for *P. infestans* (JUDELSON *et al*. 1991). If the concentration of hygromycin B used in plates on which putative

transformants were maintained did not exert a large enough selection pressure on the cultures, the transgene could have been lost in this manner.

However, it is important to note that studies in both *P. parasitica* and *P. infestans* have demonstrated that loss of transgenes do not result in a loss or reversion of gene silencing (GAULIN *et al.* 2007; VAN WEST *et al.* 1999a). Instability and loss of integrated plasmids is thus not a limiting factor in gene silencing experiments. Another possible explanation for the loss of transgenes from primary transformants concerns the coenocytic nature of *P. cinnamomi* mycelia (GAULIN *et al.* 2007). Protoplasts could thus contain multiple nuclei, of which only a fraction is transformed, resulting in heterokaryons. Single zoospore derivatives of primary transformants should be obtained to prevent loss of transformed nuclei during sub-culturing.

The negative effect of the site of chromosomal integration on transgene expression is known as the positional effect, and is well-documented in plants (KOOTER *et al.* 1999) and in oomycetes (JUDELSON and AH-FONG 2009). Repetitive segments of methylated endogenous DNA flanking the transgene can result in the spread of methylation to the transgene, resulting in gene silencing (KOOTER *et al.* 1999). Introduction of large amounts of prokaryotic sequences through integration of whole plasmids may alert methyltransferases to the presence of foreign sequences due to the unusual sequence compositions and inability of eukaryotic proteins to bind to prokaryotic sequences, resulting in methylation and gene silencing (KOOTER *et al.* 1999). In fungi, the availability of vectors allowing for homologous recombination into multiple sites has resulted in higher transformation efficiencies (RUIZ-DÍEZ 2002). Integration of plasmid DNA into heterochromatic, transcriptionally silent regions on chromosomes, such as near telomeres and centromeres which often consists of repeats, also results in an absence of foreign protein production (JUDELSON and AH-FONG 2009; PRÖLS and MEYER 1992).

The suitability of fluorescently-tagged strains of *P. cinnamomi* as tools in the study of plant-pathogen interactions should be evaluated before use in infection studies. This should involve confirmation that the transgene is stably expressed in all developmental stages of the pathogen (*in vitro*), and that expression of the transgene by transgenic strains has no significant detrimental effects on the fitness of the pathogen. Putative transformants should be compared to wild type isolates in terms of growth rate and pathogenicity. Transformed *P. capsici* strains with a reduced growth rate and virulence to pepper fruit and seedlings has been reported (DUNN *et al.* 2013), but many examples of transformants with normal growth

rates and virulence levels used in microscopy studies exist (BOTTIN *et al.* 1999; CHEN *et al.* 2009; RIEDEL *et al.* 2009; SI-AMMOUR *et al.* 2003).

It can be concluded that due to the low regeneration and success rates associated with protoplast regeneration and transformation respectively, a large number of transformation experiments have to be performed in order to obtain transformants. Isolate-specific effects on transformation efficiencies should also not be discounted, illustrated by the different results obtained with isolates 943 and TBB5T7S3. A "competence phenomena" underlying the variation in transformation efficiencies has been reported for fungi (RUIZ-DÍEZ 2002). It is suggested that the presence of subpopulations of cells or nuclei exhibiting unusually high levels of competency for DNA integration are responsible for the inconsistency observed with different protocols and different isolates. Blanco and Judelson (2005) reported that some *P. infestans* strains were not amenable to transformation through electroporation, and *P. infestans* isolates not suited to protoplast transformation have been identified based on poor protoplasting abilities (BLANCO and JUDELSON 2005; WHISSON *et al.* 2005).

A biolistics or *Agrobacterium*-based approach can be considered as alternatives to protoplast transformation of *P. cinnamomi*, as a study in the early 1990s reported on the transformation of *P. cinnamomi* with the *GUS* gene through microprojectile bombardment of intact mycelia (BAILEY *et al.* 1993). A reliable transformation protocol for *P. cinnamomi* is of utmost importance for research advances on this important pathogen. This study served to optimize the production and regeneration of protoplasts in our laboratory, and will serve as a foundation for future attempts to establish a protoplast transformation system.

Conclusion

Understanding a pathogen and the manner in which it interacts with, and manipulates its host will aid in identifying novel control strategies. In this study protoplasts were successfully released from mycelia and young germlings of *P. cinnamomi* using previously published protocols. Optimal mannitol concentrations in regeneration media were identified and regeneration of protoplasts was observed. PEG and $CaCl_2$-mediated protoplast transformation resulted in two putative transformants. No fluorescence was detected in putative transformants despite the presence of the *GFP* gene, implicating loss or silencing of the transgene. Drastically increasing the number of transformation experiments might improve chances of obtaining transformants by this method. Microprojectile bombardment

and *Agrobacterium*-mediated transformation can be considered as alternatives for protoplast transformation in the search for a reliable transformation protocol for *P. cinnamomi*.

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Tables

Table 1. *Phytophthora cinnamomi* isolates used in this study.

Isolate	Origin	Source of isolation
941	Riverside, California, USA	*Persea americana* trunk
942	Riverside, California, USA	*P. americana* trunk
943	Riverside, California, USA	*P. americana* trunk
2060	San Diego, California, USA	*P. americana* roots
TBB5T7S3	Tzaneen, Limpopo, SA	Soil in proximity to *P. americana* roots

Table 2. Comparison of sporulation ability of four different *Phytophthora cinnamomi* isolates.

Isolate	5% V8 broth		Pea broth	
	Sporangia	**Chlamydospores**	**Sporangia**	**Chlamydospores**
941*	+	+	-	-
942*	-	+	-	-
943	+	+	-	+
2060	-	+	-	+

+: Present; -: absent. *No spores were observed for isolates 941 and 942 grown in pea broth prior to induction of sporangia production through starvation of cultures.

Table 3. Gene-specific primers used to identify putative *Phytophthora cinnamomi* transformants.

Primer	Primer sequence (5'-3')	Target gene	Product size (bp)
eGFP-sense	GGCACAAGTTCTCCGTCAG	*eGFP*	295
eGFP-antisense	CGGTTCACCAGGGTATCAC		
hpt-sense	TGGAGCGAGGCGATGTTC	*Hygromycin phosphotransferase (hpt)*	484
hpt-antisense	GTGCCACCAAGCGTAAGG		

Table 4. Evaluation of growth of *Phytophthora cinnamomi* on 5% V8 agar containing increasing concentrations of the antibiotic hygromycin B.

	0 µg/ml	20 µg/ml	50 µg/ml	80 µg/ml	100 µg/ml
Repetition 1	+	+	+	-	-
Repetition 2	+	+	+	-	-
Repetition 3	+	+	+	-	-
Repetition 4	+	+	+	-	-
Repetition 5	+	+	+	-	-

Table 5. Evaluation of optimal mannitol concentration required for regeneration of *Phytophthora cinnamomi* protoplasts using protoplasts released from one-day-old cultures of *P. cinnamomi.*

	0.5 M mannitol	0.6 M mannitol	0.7 M mannitol	0.8 M mannitol	0.9 M mannitol
Nr of protoplasts (protoplasts/ml)	$4.8x10^5$	$4.8x10^5$	$4.8x10^5$	$4.8x10^5$	$4.8x10^5$
Nr of regenerated protoplasts (protoplasts/ml)	$3x10^4$	$6 x10^4$	$7 x10^4$	$4x10^4$	$3 x10^4$
% regeneration	6.25	12.5	14.5	8.3	6.25

Table 6. Evaluation of optimal mannitol concentration required for regeneration of *Phytophthora cinnamomi* protoplasts using protoplasts released from two-day-old cultures of *P. cinnamomi.*

	0.5 M mannitol	0.6 M mannitol	0.7 M mannitol	0.8 M mannitol	0.9 M mannitol
Nr of protoplasts	$10.4x10^5$	$10.4x10^5$	$10.4x10^5$	$10.4x10^5$	$10.4x10^5$
Nr of regenerated protoplasts	$5.5x10^4$	$4.5 x10^4$	$6 x10^4$	$1.5 x10^4$	0
% regeneration	5	4.32	5.7	1.4	0

Figures

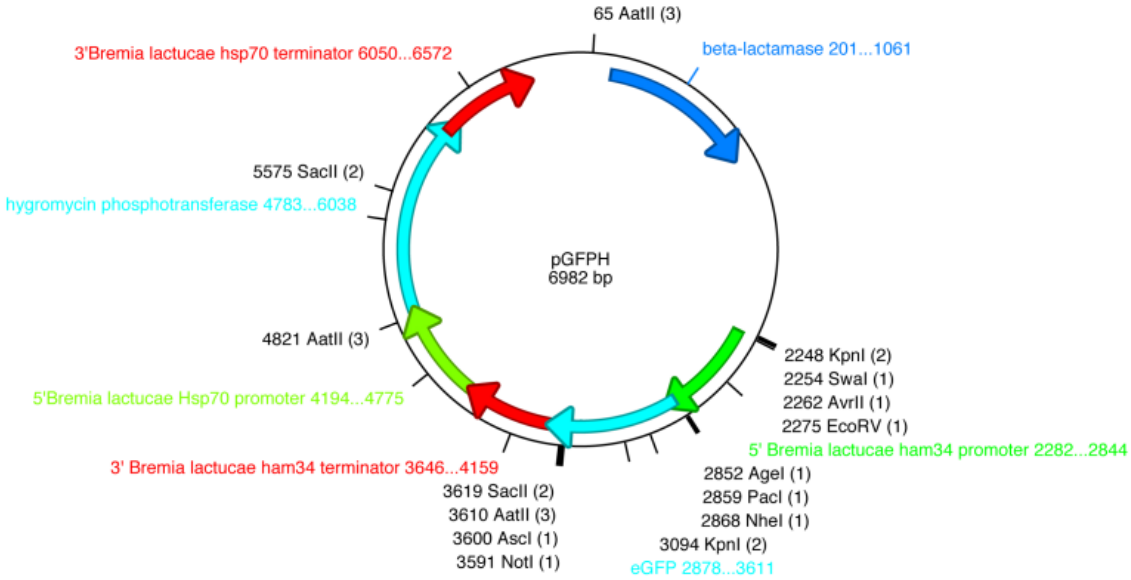

Figure 1. Plasmid map of pGFPH. Expression of the *eGFP* gene is directed by the *ham34* promoter and terminator from *Bremia lactuceae*. The *hygromycin phosphotrasferase* gene, which is controlled by *heatshock 70* promoter and terminator sequences from *B. lactuceae* serves as selectable marker. Image obtained from http://oomyceteworld.net/plasmids/pGFPH.pdf.

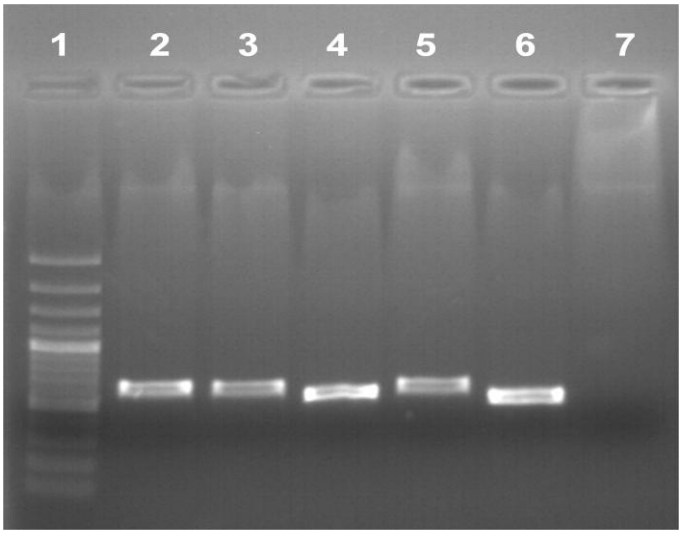

Figure 2. Agarose gel electrophoresis (1%, 80 V) of 450 bp LPV3 PCR products for identification of isolates as *Phytophthora cinnamomi*. 1: 100 bp molecular marker (O'Generuler, Thermo Scientific, Wilmington, Massachusetts, USA); 2: 941; 3: 942; 4: 943; 5: 2060; 6: TBB5T7S3; 7: negative (water) control.

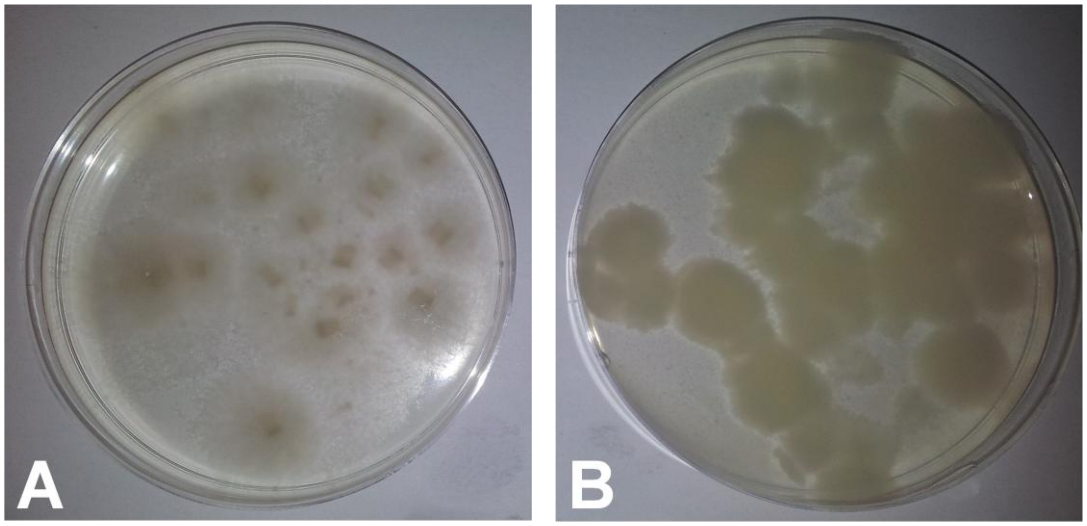

Figure 3. Culture morphology of *Phytophthora cinnamomi* in pea and 5% V8 broth. Cultures appeared clumpy in pea broth, resembling cotton wool (A), and had a more uniform appearance in 5% V8 broth (B).

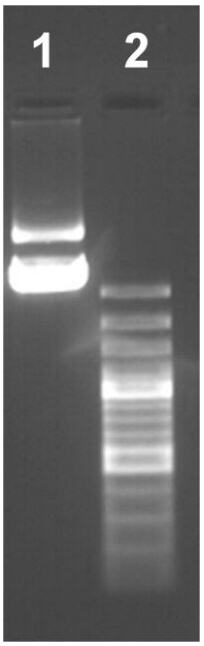

Figure 4. Agarose gel electrophoresis (1%, 80V) of pGFPH plasmid DNA. Smaller bands correspond to supercoiled plasmid DNA, and larger bands (top) to open circle or nicked plasmid DNA. 1: pGFPH; 2: 100 bp molecular marker (O'Generuler, Thermo Scientific).

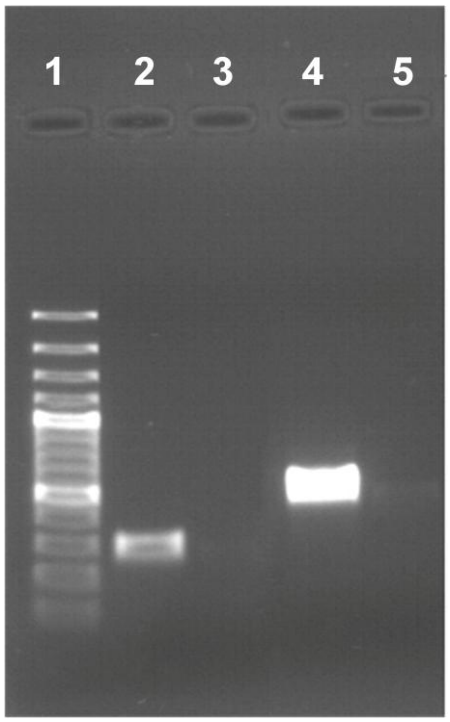

Figure 5. Agarose gel electrophoresis (1%, 80 V) of GFP and hygromycin phosphotransferase (hpt) PCR products. Lane 1: 100 bp molecular marker (O'Generuler, Thermo Scientific); lane 2: GFP PCR product (295 bp), lane 3: GFP-specific PCR negative (water) control); lane 4: hygromycin phosphotransferase PCR product (484 bp); lane 5: hygromycin phosphotransferase-specific PCR negative (water) control.

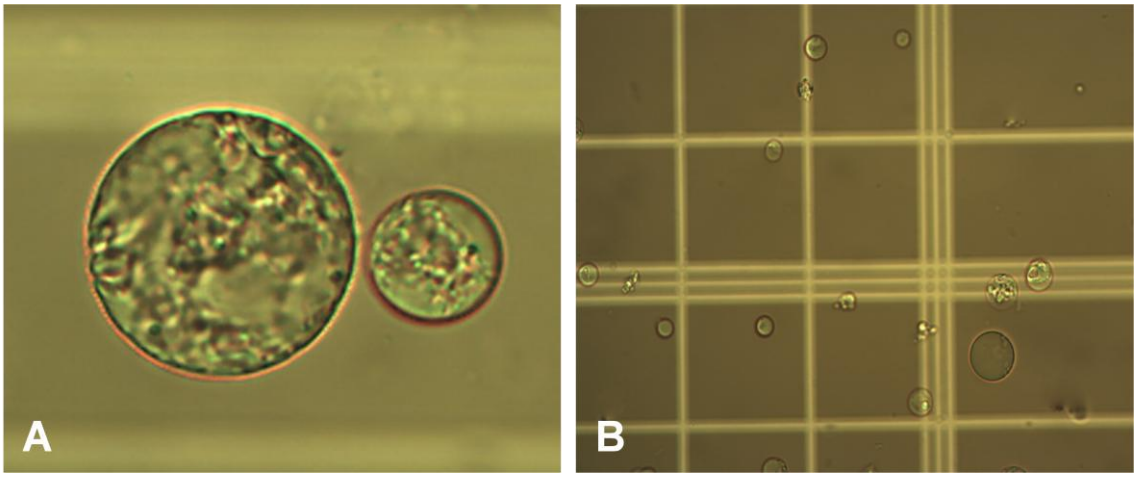

Figure 6. Stereomicroscope images illustrating variation in sizes of protoplasts obtained when digesting two-day-old mycelia of *Phytophthora cinnamomi*.

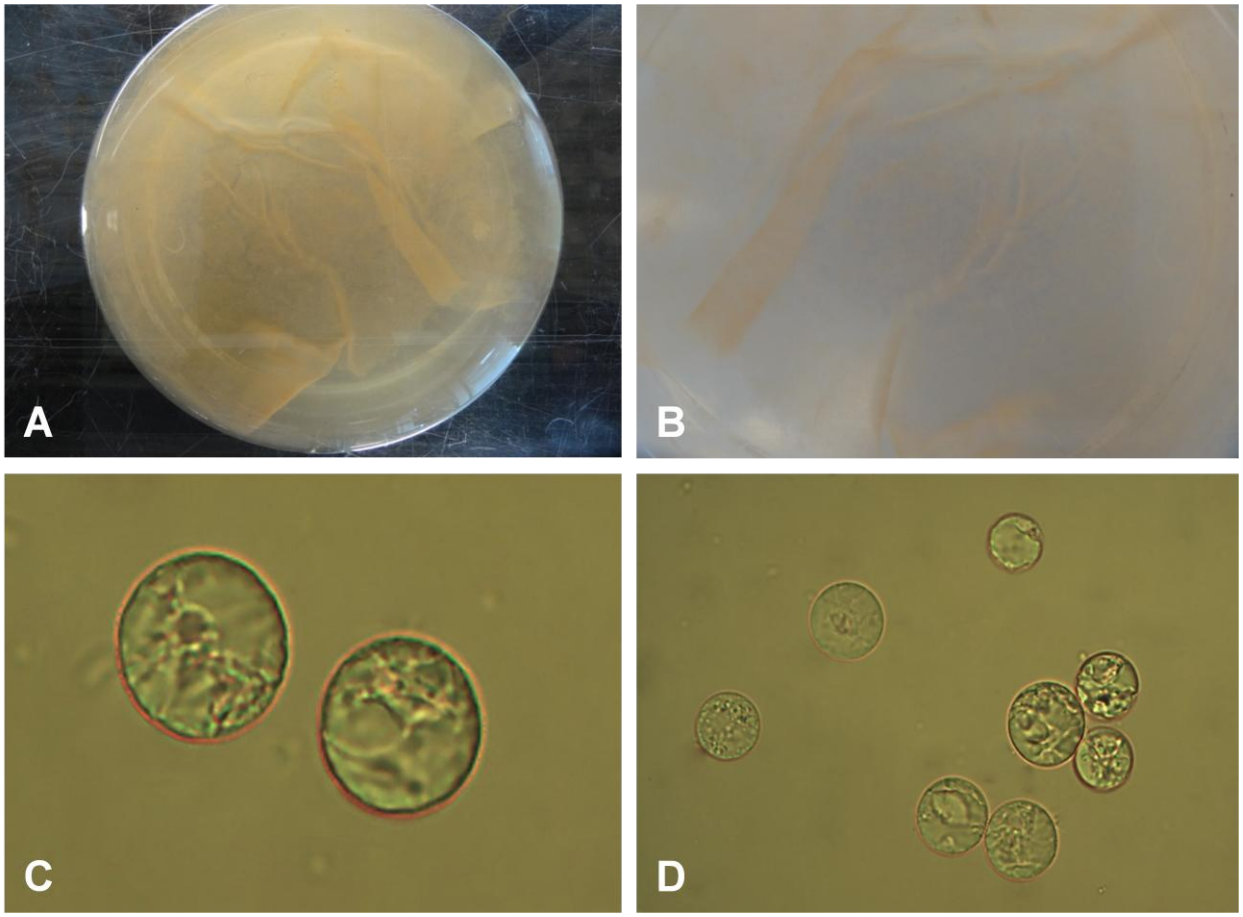

Figure 7. Young germling mycelial mats produced by allowing *Phytophthora cinnamomi* zoospores to germinate in 5% V8 broth (A and B) and protoplasts released from young germlings by digestion of cell walls (C and D). A: Flask containing mycelia mat; B: thin mycelia mat.

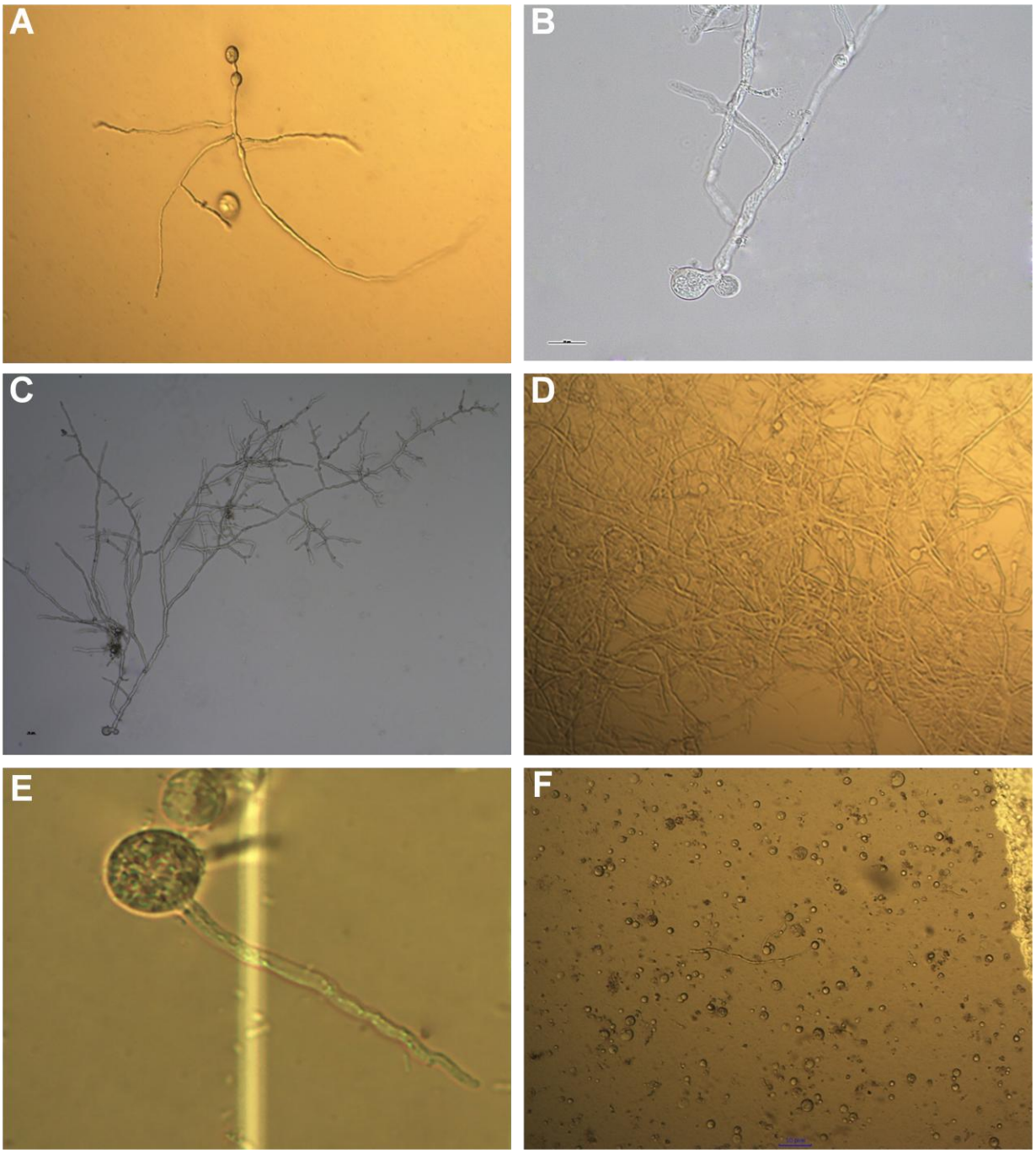

Figure 8. The effect of different concentrations of the osmoticum mannitol in regeneration media on the regeneration of cell walls and germination of protoplasts. A, B and C: growth and branching of *Phytophthora cinnamomi* protoplasts in regeneration media containing 0.5 M mannitol after 24 hr; D: protoplasts regenerated for 48 hr in regeneration media containing 0.5 M mannitol; E: shorter germtubes associated with regeneration of protoplasts in regeneration media containing higher concentrations (0.8 M) of mannitol; F: small proportion of protoplasts that regenerate.

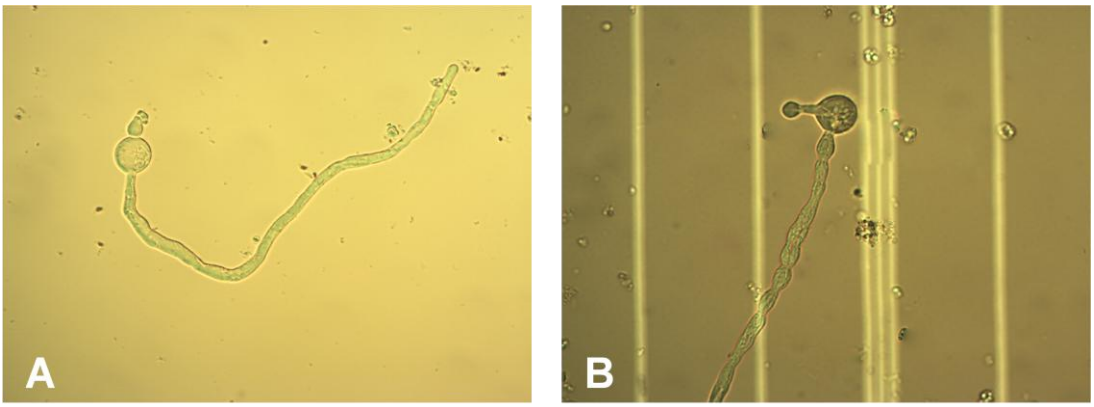

Figure 9. Protoplasts with germtubes (A) and structures resembling germinating cysts and appresoria (B) present after regeneration of protoplasts following transformation of *Phytophthora cinnamomi* protoplasts with pGFPH plasmid DNA.

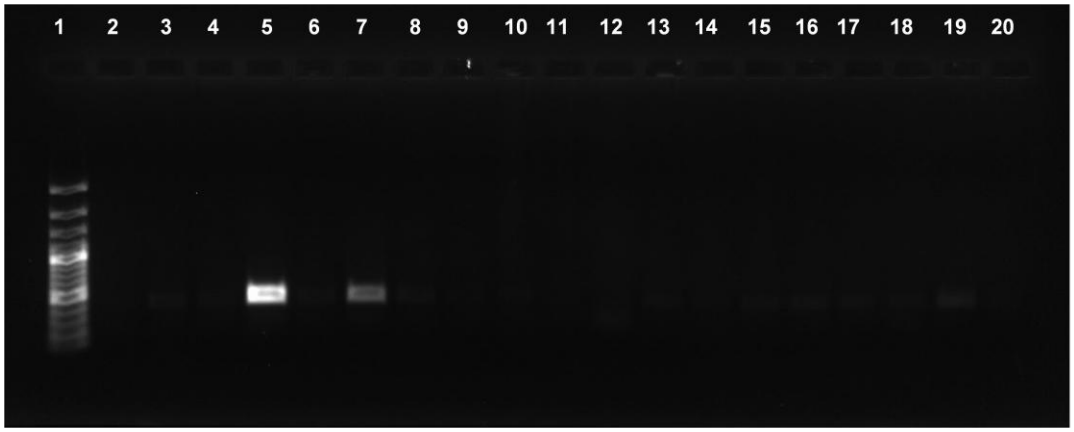

Figure 10. Agarose gel electrophoresis (1%, 80 V) of GFP specific PCR products. Colonies present on transformation selection plates were screened for the presence of the *GFP* gene. Lane 1: 100 bp molecular marker (O'Generuler); lanes 2-18: putative transformed colonies, lane 20: PCR negative (water) control.

Appendix A: Production of sporulating mycelia, release of protoplasts and PEG/$CaCl_2$-mediated transformation of *P. cinnamomi* protoplasts according to the methods described by Chen and Zentmyer (1970) and Judelson *et al*. (1991).

1. Production of sporulating mycelia (Chen and Zentmyer (1970))

- Grow isolate on 10% V8 agar for 3-4 days at 25°C in the dark
- Using a scalpel, cut agar into very small (1 mm^2) pieces, and inoculate approximately 20 ml 5% V8 broth containing 20 mg/l β-sitosterol in a 90 mm petri dish with 5-10 agar blocks
- Incubate plates under fluorescent lights for 2-3 days until a mycelia mat covers most of plate at room temperature
- Wash cultures four times with mineral salt solution, and incubate for two days in approximately 10 ml mineral salts solution under fluorescent light.

It is important that cultures are not completely covered by mineral salt solution, as aeration improves sporulation. Monitor cultures for the presence of sporangia, as sporulation usually reaches a maximum after 24-36 hours. Cultures are exposed to a brief cold shock to stimulate release of zoospores.

- Wash plates twice with 4°C dH_2O, and incubate plates at 4°C for 30 min, followed by incubation at room temperature for 60-90 min to allow for zoospore release (monitor release of zoospores under the microscope)
- Harvest zoospores through a 15 µm nylon mesh, and incubate for 24 hr in 5% V8 broth amended with 50 µg/ml ampicillin, 50 µg/ml vancomycin and 4 ml/l nystatin in a flask in the dark

A final concentration of approximately 2-5x10^5 zoospores/ml are required for the formation of a thin, uniform mat of young germlings. If lower numbers of zoospores are obtained, zoospores can be concentrated by centrifugation as encystment will also occur during germination of zoospores. Flasks should not be filled to more than 10% of their total volume to allow for sufficient aeration

2. Protoplast production and transformation

(The following section will be described briefly, as it is identical to the modified protocol described by Judelson *et al* (1991) with the exception of a few side comments. Protocol available at http://oomyceteworld.net)

- Harvest germlings by pouring V8 broth through a 50 µm nylon mesh
- Pick mycelia up from membrane with forceps, and place in a 50 ml tube, and rinse with 40 ml FPB buffer
- Add enzyme digesting solution, and incubate for 40 min at room temperature

After approximately 10 minutes' incubation, gently mix contents of tube again

- To harvest protoplasts, pass enzyme digestion solution through a 50 µm nylon membrane

Approximately half of protoplasts are lost during washing steps, thus approximately 2-3x10^7 protoplasts are ideal at this point to allow for one transformation

- Spin protoplasts in a centrifuge with a swinging bucket rotor at 700xg for 4 min to harvest. Based on preliminary protoplast counts, add DNA to Lipofectin to form liposomes in preparation for transformation

On occasion I have spun protoplasts at 950xg in attempts to minimise protoplast loss during washing steps, with no apparent negative effect on protoplasts

- Resuspend pelted protoplasts in FPB, respin, pour of supernatant
- Resuspend in 15 ml FPB with 15 ml MT, respin, pour of supernatant
- Resuspend in 30 ml MT + 10 mM $CaCl_2$, remove an aliquot to determine protoplast yield using a hemocytometer, respin, pour of supernatant
- Resuspend protoplasts in MT + 10 mM $CaCl_2$ to a final volume of 1-3x10^7 protoplasts/0.7 ml
- Add protoplasts to DNA mixture, mix gently by rolling tube, and incubate for 4 min
- Add an equal volume of 50% PEG-MW3350 (25 mM $CaCl_2$ and 10 mM Tris pH 7.5) by gently rolling tube while adding, and invert once. Incubate 4 min
- Add 2 ml 5% V8 broth + 0.7 M mannitol, and invert once. Add 8 ml 5% V8 broth + 0.7 M mannitol, and invert once
- Add to a tube containing 25 ml 5% V8 broth + 0.7 M mannitol containing 50 µg/ml ampicillin, 50 µg/ml vancomycin and 4 ml/l nystatin
- Incubate overnight at room temperature
- The following day, invert tube, and remove an aliquot to monitor protoplast regeneration under a microscope
- Harvest regenerated protoplasts by centrifugation at 1000xg, pour of supernatant, leaving 2-3 ml of broth behind. Gently resuspend protoplasts in this liquid, and plate 0.5 ml aliquots on 5% V8 agar containing the appropriate concentration of the antibiotic (determined previously for each individual isolate)
- Incubate plates at 25°C in the dark for colonies to appear

Chapter 3

Evaluation of variance in pathogenicity of *Phytophthora cinnamomi*

Abstract

Phytophthora Root Rot (PRR) caused by *Phytophthora cinnamomi* is the major threat to sustainable and industrious avocado production in South Africa. The amount of variation in virulence within pathogen populations has a significant impact on commercial breeding programmes aimed at producing resistant plant material. Durability of resistance depends on variation in virulence in pathogen populations, as well as specific host-pathogen interactions. A re-evaluation of the amount of variation in virulence, with a focus on isolates from avocado orchards was required to aid breeding programmes focussed on the production of resistant avocado rootstocks. The aim of this study was to assess differences in growth rate, sporulation and virulence in 12 *P. cinnamomi* isolates from different geographical locations. Isolates were grown on three different culture media to evaluate growth rate, and zoospores were produced in axenic culture to assess sporulation. Stem inoculation of avocado was used to determine differences in virulence between isolates by evaluating lesion lengths and quantifying the amount of pathogen DNA *in planta*. Significant differences in growth rate, sporulation and virulence to avocado were observed, and geographical origin of isolates had no effect on pathogenicity. A single isolate or subset of isolates did not perform well across all experiments. Some isolates exhibited high levels of sporulation and virulence to avocado, but were amongst the slowest growers. Other isolates grew and sporulated well, but displayed average levels of virulence. A subset of isolates that grow and sporulate well, are more virulent to avocado and which are good colonizers were identified for use as a mixed inoculum in avocado infection studies and screening trials. Results of this study can contribute to future screening trials for more tolerant or resistant avocado rootstocks by emphasizing the important role the selection of isolates can play.

Introduction

Phytophthora cinnamomi Rands is an economically-important, soil-borne, wide host range oomycete which has a devastating impact on both economically important crop species and native vegetation (HARDHAM 2005; VON BROEMBSEN 1984). In South Africa, *P. cinnamomi* is of particular importance to the avocado (*Persea americana* Mill.) industry as Phytophthora root rot (PRR) results in significant yield losses annually. Currently, control of this pathogen and PRR relies mainly on the use of avocado rootstocks tolerant to *P. cinnamomi* infection, good farming practices and chemical control through the use of phosphite trunk injections (AKINSANMI and DRENTH 2013; DARVAS *et al.* 1984; ESHRAGHI *et al.* 2011). However, despite the economic significance of this disease world-wide, the mechanism underlying tolerance or susceptibility in avocado rootstocks is still unknown. This complicates breeding and screening of avocado rootstocks, as specific markers associated with tolerance or susceptibility are unavailable.

The threat of the possible evolution of new *P. cinnamomi* genotypes which are resistant to phosphites (DOBROWOLSKI *et al.* 2008), and the many negative effects associated with conventional farming practices highlights the need for the integration of plant pathology and plant breeding to produce superior disease resistant crops (LI *et al.* 2013). Pesticides are harmful to both the environment and human health, and its use places a selection pressure onto pathogen populations for new genotypes with reduced sensitivity to these chemical control agents. A well known example is the emergence of *Phytophthora infestans* strains resistant to metalaxyl (DAVIDSE *et al.* 1981; WANG *et al.* 2012). It has also been shown that *P. cinnamomi* isolates from phosphate-treated avocado orchards are better able to infect plant material, and are less sensitive to, plant material sprayed with phosphite when compared to isolates from untreated orchards or native vegetation (DOBROWOLSKI *et al.* 2008). There has been a recent shift from conventional to organic agriculture, which mainly relies on pathogen-resistant crops and management strategies to limit disease (LI *et al.* 2013).

Some organic management strategies cannot be used in avocado production. For example, crop rotation and biological control are not feasible options to control *P. cinnamomi* in avocado orchards. Avocado trees are not annual crops, requiring almost a decade to become productive, after which they can produce fruit for decades, eliminating the possibility of crop rotation. A number of possible biocontrol agents such as *Myrothecium roridum* Tode (GEES and COFFEY 1989), *Micromonospora* spp. (EL-TARABILY *et al.* 1996), *Trichoderma* spp., *Gliocladium virens* J. H. Mill., Giddens & A. A. Foster (CHAMBERS and SCOTT 1995)

and *Penicillium* spp. (FANG and TSAO 1995) have been identified for *P. cinnamomi*, but practical implementation is also hindered by the perennial nature of this crop. For perennial crops and soilborne pathogens such as *P. cinnamomi* it is difficult to establish and maintain large enough biocontrol populations to successfully inhibit disease. Mulching is effectively employed in avocado orchards, and improves drainage and the general health of the rhizosphere (HARDHAM 2005). The limited worth of alternative control strategies thus highlights the fact that sustainable avocado production in the future will have to rely heavily on the availability of resistant or highly tolerant rootstocks.

An understanding of the amount of variation in virulence among *P. cinnamomi* isolates is important for avocado rootstock breeding programmes. This may influence the selection of isolates used in screening trials for breeding programmes aimed at producing tolerant or resistant rootstocks. The durability of resistance in crops is dependent on both the amount of variation in the pathogen population and also the host-isolate interaction (LINDE *et al.* 1999b; ROBIN and DESPREZ-LOUSTAU 1998). Variation with regard to virulence exists in *P. cinnamomi* populations, despite the limited amounts of genetic diversity reported in the past (DOBROWOLSKI *et al.* 2003; LINDE *et al.* 1997; LINDE *et al.* 1999a; OLD *et al.* 1988; OLD *et al.* 1984).

In a study conducted on a *P. cinnamomi* population collected from various hosts in the Cape province and Mpumalanga by Linde *et al.* (1999), significant differences in both *in vitro* growth rate and virulence to *Eucalyptus smithii,* and a positive correlation between growth rate and virulence were observed (LINDE *et al.* 1999b). Variation in virulence on a number of different tree species was also reported for a collection of *P. cinnamomi* isolates from France (ROBIN and DESPREZ-LOUSTAU 1998) and New Zealand (CHEE and NEWHOOK 1964). A study on the diversity of 16 *Phytophthora capsici* isolates from Spain revealed three random amplified polymorphic DNA (RAPD) groups, and isolates showed variation in levels of virulence on four pepper lines (*Capsicum annuum* L.) with varying levels of susceptibility (SILVAR *et al.* 2006). Some isolates were hyper virulent on the most susceptible cultivars, while other isolates were hypo virulent on the same cultivars. No correlation was found between virulence and RAPD genotype. A similar study on 93 *P. infestans* isolates from Poland used three SSR (Simple Sequence Repeat) markers, lesion size on leaflets and virulence factors to assess genetic diversity and virulence respectively (SLIWKA *et al.* 2006). The SSR markers revealed high levels of genetic diversity among the isolates, as all three markers were polymorphic, and had three to five alleles at a locus. Isolates were divided into three virulence classes, weak, moderate and high, but moderate and high virulence levels predominated.

The most recent studies on the amount of variation in pathogenicity and genetic diversity among South African *P. cinnamomi* isolates were conducted in the 1990's, and only a small fraction of isolates used in those studies originated from avocado orchards (LINDE *et al.* 1997; LINDE *et al.* 1999a). As seen from the above mentioned examples, variation in virulence and other phenotypic traits such as *in vitro* growth rate and resistance to chemicals occur in both genetically diverse (*P. infestans* in Poland) and more clonal (*P. cinnamomi* in South Africa and *P. capsici* in Spain) populations. Clonality in pathogen populations does not imply that all individuals are identical (DOBROWOLSKI *et al.* 2003). Re-evaluation of differences in phenotypic traits such as growth rate, sporulation and virulence is thus required for *P. cinnamomi* isolates from avocado in South Africa.

The aim of this study was to assess the variation in virulence components such as growth, sporulation and virulence to avocado in *P. cinnamomi* isolates collected from avocado orchards in three provinces in South Africa and in California, United States of America. The influence of geographic origin of the isolates, and correlations between growth and sporulation rates and virulence were investigated.

Materials and Methods

Isolates

Twelve isolates recovered from avocado orchards in various locations were used in all tests in this study **(Table 1)**. Isolates 941, 942, 943, 435 and 2060 were kindly provided by Dr Akif Eskalen from the Department of Microbiology and Plant Pathology at the University of California, Riverside, USA. Isolates of South African origin were isolated from either diseased avocado feeder roots or baited from soil samples collected in close proximity to avocado trees. Root sections were surface sterilized with 70% ethanol, rinsed in dH_2O, and plated on selective NARPH cornmeal agar (CMA) (17 g cornmeal agar (Sigma-Aldrich, Taufkirchen, Germany), dH_2O to 1000 ml, 50 mg hymexazol, 10 mg rifampicin, 250 mg ampicillin and 125 mg a.i. pentachloronitrobenzene). For baiting of soil samples, small (1 cm^2) blocks of avocado leaves were surface sterilized in 70% ethanol, and placed in polystyrene cups containing the soil samples and approximately 150 ml of dH_2O. Soil samples were incubated for three to seven days to allow *P. cinnamomi* zoospores to infect avocado leaves, after which avocado leaves were plated onto NARPH CMA. *Phytophthora cinnamomi* was isolated from selective media based on culture morphology using a light microscope, and single hyphal tipped onto half strength potato dextrose agar (PDA) (19.5 g

potato dextrose agar (Biolab, Merck), 7.5 g agar (Difco, BD Diagnostic Systems, Maryland, USA) in 1000 ml dH_2O) to obtain pure cultures. Cultures were regularly maintained in the laboratory on 5% V8 agar (50 ml cleared V8 juice (Campbell Soup Company, USA), 0.5 g $CaCO_3$, 18 g agar (Difco) and 1000 ml dH_2O) at 25°C in the dark. All isolates used in this study are maintained in the culture collection of the Fruit Tree Biotechnology Programme at the University of Pretoria, South Africa.

DNA extraction from *P. cinnamomi*

Cultures were grown on 5% V8 agar plates at 25°C for one week prior to DNA extraction. Aerial hyphae were scraped from the plates using a needle, and DNA was extracted as described in Chapter 2. Concentrations and purity of the extracted DNA was measured using a NanoDrop ND-1000 spectrophotometer and NanoDrop 3.2.1 Software (Nanodrop Technologies, Inc., Montchanin, Delaware, USA). DNA was analyzed by agarose gel electrophoresis (2%), and visualized under UV light through the addition of GelRed (Biotium Inc., California, USA).

Identification of *P. cinnamomi* isolates using molecular techniques

The identity of all isolates was confirmed using a *P. cinnamomi* species-specific LPV3 primer set for Polymerase Chain Reaction (PCR) (KONG *et al.* 2003), as well as sequencing of the *ITS* (internal transcribed spacer) gene region. The LPV3 species-specific PCR was performed as previously described. The 20 µl ITS PCR reactions contained 30-50 ng template DNA, 2 µl of 10x NH_4 buffer (Bioline, London, United Kingdom), 1.5 µl of 50 mM $MgCl_2$ (Bioline), 200 µM of each dNTP, 0.2 µM of the sense primer ITS6 (5' GAAGGTGAAGTCGTAACAAGG 3') (COOKE and DUNCAN 1997), 0.2 µM of the antisense primer ITS4 (5' TCCTCCGCTTATTGATATGC 3') (WHITE *et al.* 1990) and 1 unit of BIOTAQ DNA Polymerase (Bioline). The PCR program consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, and a final extension step at 72°C for 10 min. A 2720 Thermal Cycler (Applied Biosystems) was used for the PCR. PCR products were analyzed by agarose gel electrophoresis (2% agarose gel, 80 V) and visualized under UV light. ITS PCR products were purified for sequencing using Exo-Sap (Exonuclease 1-Shrimp alkaline phosphatase) (Fermentas Life Sciences, Hanover, USA) by adding 8 µl of Exo-Sap to each 20 µl of ITS PCR product. This was followed by two 15 min incubation steps, first at 37°C, followed by incubation at 80°C.

Each 12 µl sequencing reaction consisted of 2 µl template DNA, 2 µl of BigDye Terminator v3.1 Ready Reaction Mix, 1 µl of 5x Sequencing buffer and 1 µM of either the ITS6 or ITS4 primer for the forward and reverse reactions respectively.

Sequence alignment and phylogenetic analysis

ITS sequences obtained for all isolates were used in a BLASTn search in the National Centre for Biotechnology Information (NCBI) database to identify isolates (BENSON *et al.* 2013). Isolates were also subjected to phylogenetic analysis to more accurately confirm their identity. Forward and reverse raw sequence reads were assembled in CLC Bio Main workbench v.6 (CLC Bio, www.clcbio.com). Consensus sequences of each isolate were manually edited and aligned in MEGA 5 (TAMURA *et al.* 2011) using Muscle. Maximum Parsimony (MP) analysis of data in PAUP v. 4.0 (SWOFFORD 2002) was used for tree construction. Trees were obtained using the heuristic search option with 1000 replicates, random addition of sequences, tree bisection reconnection (TBR) branch swapping strategy, and gaps were treated as a 5th character. Statistical support for branches was obtained using 1000 replicates of non-parametric bootstrap analysis of the sequence data. Sequences of *P. cinnamomi, Phytophthora cinnamomi* var *cinnamomi, Phytophthora cambivora, Phytophthora carpensis, Phytophthora citricola, Phytophthora multivora, Phytophthora frigida* and *Phytophthora cryptogea* obtained from the NCBI were included in phylogenetic analysis. *Pythium vexans*, *Pythium ultimum* var *ultimum* and *Pythium aphanidermatum* were used as outgroups for rooting of trees. Trees were modified in MEGA 5.

Recovery of virulence of isolates in apple tissue

Isolates in this collection have been cultured for varying time periods, thus isolates were used to infect plant material and re-isolated in a bid to recover their virulence. All isolates used in this study were passaged through Granny Smith apples, as previously described for *P. cinnamomi* (KING *et al.* 2010; RIBEIRO 1978). Apples were surface sterilized with 70% ethanol, after which small blocks of apple were excised. Mycelial plugs consisting of the isolates growing on 5% V8 agar were placed inside the apple, where after the excised apple section was replaced. Apples were wrapped in a double layer of cling wrap, and incubated for ten days at 20°C in the dark. This was followed by re-isolation of *P. cinnamomi* from the apple onto NARPH selective 5% V8 agar. Sections of diseased apple were plated onto

NARPH 5% V8 media, and incubated for three days at 25°C in the dark. *Phytophthora cinnamomi* isolates were identified based on morphology using a light microscope, and transferred to 5% V8 agar for DNA extraction and subsequent identification using the LPV3 species-specific PCR, as described above.

Assessment of sporulation ability of *P. cinnamomi* isolates

Sporulating mycelia were produced *in vitro*. For each isolate, 90 mm petri dishes containing 20 ml of 5% V8 agar were inoculated with the respective isolates and incubated at 25°C for five days in the dark. An 8 mm cork borer was used to excise mycelial discs from the colony margin. Each plug was placed in a 90 mm petri dish containing 20 ml of dH_2O, and incubated at 25°C under fluorescent lights for four hours. After the four hour incubation, the dH_2O was replaced, and the plates were incubated at 25°C under fluorescent lights overnight. Zoospores were released from sporulating mycelia through cold shock. The dH_2O was removed, and replaced with 1 ml of dH_2O (4°C), followed by incubation of plates at 4°C for 30 min. After completion of cold shock treatment, plates were incubated at room temperature for 90 min to allow for zoospore release. Zoospores were collected using a 15 µm nylon mesh (Biodesign, New York, USA), and zoospore yield was determined using a haemocytometer (Marienfeld Laboratory Glassware, Lauda-Königshofen, Germany). Each experiment contained at least three technical repeats, and the experiment was performed three times.

Evaluation of *P. cinnamomi* growth rate *in vitro*

The growth rate of 12 isolates was evaluated *in vitro* on half strength PDA (Biolab), 5% V8 agar and MEA (malt extract agar) (Biolab, Merck) at 25°C. Isolates were grown on 90 mm petri dishes containing 10 ml of half strength PDA for six days at 25°C in the dark prior to inoculation. An 8 mm diameter cork borer was used to excise mycelial discs from the colony margins, and mycelial plugs were transferred to the centre of 90 mm petri dishes containing 20 ml of the respective culture media. Five petri dishes were inoculated for each isolate on each culture media. Plates were incubated in a randomized block design at 25°C for four days, and colony diameter was measured daily.

Virulence of *P. cinnamomi* isolates on avocado trees

Three-day-old cultures of each isolate grown at 25°C in the dark on 90 mm petri dishes containing 10 ml 5% V8 agar were used to inoculate stems of two year old Dusa® avocado trees. A large cork borer (8 mm diameter) was used to remove bark and wound stems. Mycelial plugs were generated in the same manner as described above, using a 7 mm cork borer to excise mycelia disks from the colony margins, and were applied to stem wounds. Inoculation with sterile 5% V8 agar mycelia plugs served as controls, and inoculated wounds on stems were sealed with Parafilm™ (Pechiney Plastic Company, Illinois, USA). Five avocado trees were inoculated per *P. cinnamomi* isolate, with two stem inoculations per tree. Avocado trees were maintained in a randomized block design in the greenhouse at 25°C, and watered two to three times per week. Lesion lengths (mm) on stems were measured five weeks post-inoculation after removing the bark with a scalpel.

DNA extraction from avocado stems

Genomic DNA was extracted from infected Dusa® avocado stems using a protocol based on a method for RNA extraction from *Pinus* spp. (CHANG *et al.* 1993), and modified for DNA extraction from plants (BRUNNER *et al.* 2001; MCNICKLE *et al.* 2008). Approximately 500 mg of wet avocado stem material was ground to a fine powder in a IKA A11 basic analytical mill (United Scientific (Pty) Ltd., San Diego, California, USA) using liquid nitrogen, and 15 ml extraction buffer (100 mM Tris-HCl pH 8, 25 mM ethylenediaminetetraacetic acid (EDTA) (Merck), 25mM • 2M NaCl, 2% (w/v) hexadecyltrimethylammonium bromide (CTAB) (Merck), 2% (w/v) polyvinylpyrrolidone K30 (PVP) (Sigma-Aldrich), 500mg/L spermidine, 2% (v/v) 2-mercaptoethanol, 5% (w/v) polyvinylpolypyrrolidone (PVPP)) added to each sample. Samples were mixed by vortex, and incubated at 65°C for 30 min. Cell debris was removed by centrifugation at 4 000 rpm for 20 min. Supernatant was collected and two chloroform extractions performed through addition of 16 ml of chloroform and centrifugation at 4 000 rpm for 20 min. One and a half volumes of chilled isopropanol were added to supernatant, followed by incubation of samples at -20°C overnight. Samples were centrifuged at 4 000 rpm for 1 hr, followed by washing of pellets with 70% ethanol. Pellets were air dried for 30 min, and resuspended in 50 µl TE buffer. DNA was purified by two subsequent phenol/chloroform (1:1) extractions, and recovered using standard ethanol precipitation. DNA concentrations were adjusted to 20 ng/µl for qPCR.

Koch's postulates and pathogen quantification using a nested qPCR protocol

Following completion of the pathogenicity trial on avocado trees, the presence of *P. cinnamomi* in diseased avocado stems was confirmed with a conventional nested LPV3 PCR protocol, using an outer and inner primer pair **(Table 2)** directed against the Lpv storage protein expressed in *P. cinnamomi* zoospores (ENGELBECHT *et al.* 2013; KONG *et al.* 2003). Two subsequent rounds of conventional PCR allowed for the detection of relatively low levels of pathogen DNA amongst plant DNA. The first round of PCR (outer LPV3 PCR) was performed as described in Chapter 2 for the identification of isolates as *P. cinnamomi*, with the exception that the number of PCR cycles was decreased to 15. In the second round of PCR (inner LPV3N PCR), a 77 bp fragment of the outer LPV3 PCR product was amplified. The inner LPV3N PCR was performed in the same manner as described for the LPV3 PCR, using 2 µl of the first PCR product as template, and the primer pair LPV3N-sense and LPV3N-antisense (ENGELBECHT *et al.* 2013). The amount of pathogen in diseased avocado stem material was determined by comparing the amount of pathogen and plant DNA in each sample using quantitative PCR (qPCR). Pathogen quantification in diseased stems was performed using the method described by Engelbrecht *et al.* (2013). A nested LPV3 PCR protocol, as described above was used for pathogen quantification, with the exception that the second round of PCR amplification was performed by qPCR. For quantification of *P. cinnamomi* DNA in DNA extracted from stem material, each 10 µl qPCR reaction contained 5 µl Sensimix™ SYBR No-ROX Kit (Bioline), 10 µM each of sense and antisense primers (LPV3N sense and antisense), and 0.5 µl PCR product from first round conventional LPV3 PCR as template. Quantitative PCR was performed on a Bio-rad® CFX 96 instrument (California, USA), and cycling conditions consisted of 10 min at 95 °C (hot start pre-incubation) followed by 40 cycles of 5 sec at 95°C, 5 sec at 60°C and 5 sec at 72°C. Quantification of the avocado *actin* gene using actin-sense and actin-antisense primers (ENGELBECHT *et al.* 2013) was performed in 10 µl reactions containing 5 µl Sensimix™ SYBR No-ROX Kit (Bioline), 10 µM each of sense and antisense primers, and 2 µl template DNA. The PCR program for quantification of the *actin* gene consisted of pre-incubation for 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 15 sec at 60°C and 15 sec at 72 °C. The amount of plant and pathogen genomic DNA present in each sample was calculated based on standard curves constructed using known amounts of pathogen and plant DNA, in the same manner as described for quantification of the *actin* and *Lpv* genes respectively (ENGELBECHT *et al.* 2013). Negative PCR controls contained dH_20. For each isolate, a minimum of three biological replicates were included, and all reactions were performed in

triplicate. Inner LPV3N and actin PCR products were analyzed by agarose gel electrophoresis (1% agarose gel, 80 V) and visualized under UV light.

Statistical analysis

JMP '9 software (SAS, North Carolina, USA) was used to analyze data generated by the growth, sporulation and pathogeniciy trials, as well as qPCR data. This involved analysis of variance (ANOVA), followed by a Least Square Means Differences Students' t-test at a significance level of $p>0.05$. Amplification data from the serial dilutions of pure *P. cinnamomi* and avocado DNA was used to calculate standard regression curves for the LPV3N and actin qPCRs 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) (ENGELBECHT *et al.* 2013).

Results

DNA extraction and identification of isolates using molecular techniques

Genomic DNA was successfuly extracted from pure cultures of *P. cinnamomi*. The identity of 12 isolates selected for this study was confirmed as *P. cinnamomi* using a species-specific LPV3 PCR (KONG *et al.* 2003) and the sequence of the *ITS* gene region **(Figure 1)**. The LPV3 primer pair amplified a 450 bp PCR product, and the ITS4/6 primer pair a fragment in the range for *Phytophthora* spp (826 bp to 941 bp), as expected. ITS sequences of all isolates showed significant homology (BLASTn E-values of 0.0) to *P. cinnamomi* sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/). Isolate 435, an isolate obtained from the USA was identified as *Pythium* spp. after failure to amplify the LPV3 fragment and BLASTn analysis of ITS sequence data.

Sequence alignment and phylogenetic analysis

Two parsiminious trees were produced by MP analysis of ITS sequence data from 14 *P. cinnamomi*, two *P. cinnamomi* var *cinnamomi*, one *P. cambivora*, two *P. carpensis*, two *P. citricola*, two *P. multivora*, two *P. frigida*, two *P. cryptogea*, one *Pythium ultimum* var *ultimum*, one *Pythium vexans* and one *P. aphanidermatum* sequence. The consistency and retention indexes were 0.858 and 0.876 respectively. Bootstrap values (BS) are indicated on branches **(Figure 2)**, and were significant (>70%) for all branches except one. Parsimony analysis

confirmed identity of isolates used in this study as *P. cinnamomi*, as ITS sequences of isolates grouped with known *P. cinnamomi* ITS sequences obtained from NCBI.

Recovery of virulence of isolates in apple tissue

Following a 10 day incubation period at 20°C, all apples showed internal disease symptoms. Symptoms included brown water soaked lesions and tissue collapse. Some isolates such as KB4T8R1 and FVB6T2S4 caused severe internal and external disease symptoms, while symptoms were relatively mild for other isolates such as GKB4T9S4 and KB12T4R3, for which no external symptoms were observed **(Figure 3)**. All 12 isolates were successfully recovered from diseased apple material, as confirmed by successful amplification of 450 bp LPV3 *P. cinnamomi*-specific PCR fragments from DNA extracted from isolates re-isolated from apple material **(Figure 4)**.

Assessment of sporulation ability of *P. cinnamomi* isolates

Based on average zoospore production over three independent experiments FVB6T2S4, GKB4T9S4 and HB2T12R1 were identified as high sporulating isolates when compared to other isolates **(Figure 5)**, but no significant differences in zoospore production were observed between the three isolates. On average, TBB5T7S3 and HB8T12R1 produced the smallest number of zoospores, and the number of zoospores produced was significantly lower when compared to FVB6T2S4, GKB4T9S4 and HB2T12R1. KB13T4R3, HB6T3S1, KB4T8R1, 941, 942, 943 and HB5T11R2 produced intermediate numbers of zoopores which were not significantly different from one another. Zoospore production was inconsistent between experiments, and no isolates consistently produced the highest or lowest number of zoospores in individual experiments.

Evaluation of *P. cinnamomi* growth rate *in vitro*

All isolates grew in a radial shape within the agar on 5% V8 plates, and in a characteristic rosette or scalloped pattern on half strength PDA and MEA as expected **(Figure 6)**. The 12 isolates showed variation in both the growth rates of different isolates on the same culture media, as well as differences between the same isolate on different culture media. Significant differences ($P<0.05$) in the amount of growth were observed between isolates on

each culturing media. On the 5% V8 agar media, the average colony diameter ranged from 48.7 mm (KB4T8R1) to 73.6 mm (HB2T12R1) **(Figure 7)**. On the half strength PDA, the variation in colony diameter was smaller (9 mm in comparison to 24.9 mm observed on 5% V8 media), with a minimum of 48.4 and a maximum of 57.4, with the same two isolates constituting the fastest and the slowest growers **(Figure 8)**.

For the 5% V8 agar and half strength PDA, geographic origin of the isolates did not influence growth, as no correlation between the amount of growth and origin was observed, with isolates from all locations scattered throughout the distribution. On MEA, isolate 942 was the slowest grower, with an average colony diameter of 43.4 mm after four days, and HB8T12R1 was the fastest grower, with an average colony diameter of 55.3 mm **(Figure 9)**. Growth rates of all isolates on all nutritional media were linear. On the 5% V8 agar, isolate HB2T12R1 grew significantly faster than all other isolates throughout the four day time period **(Figure 10)**. On MEA, HB8T12R1 grew faster than the other isolates between two and a half and four days **(Figure 11)**, and on half strength PDA HB2T12R1 had a higher growth rate compared to the other isolates between one and a half and four days **(Figure 12)**. The growth rate of HB8T12R1 decreased slightly after three and four days post inoculation on MEA.

Virulence of *P. cinnamomi* isolates on avocado

Inoculation with all 12 isolates resulted in the development of lesions on avocado stems. Development of lesions was not observed on control plants, as expected **(Figure 13 A)**. Lesion length ranged from 33 mm for TBB5T7S3 **(Figure 13 B)** to 66 mm for HB5T11R2 **(Figure 13 C)**. HB5T11R2 appeared to be the most virulent, as the average lesion length produced was significantly larger than what was observed for all other isolates, except GKB4T9S4 **(Figure 14)**. Although TBB5T7S3 resulted in the smallest lesion size, lesion length was not significantly smaller than those caused by isolates 941, 942, 943, KB4T8R1 and HB6T3S1. The standard deviation calculated for data of each isolate ranged from 8.37 to 38.25.

Koch's postulates and pathogen quantification using a nested qPCR protocol

Amplification of a 77 bp fragment of the *Lpv* gene in a conventional nested PCR using the LPV3N species-specific primer pair confirmed the presence of *P. cinnamomi* in diseased

avocado stem material **(Figure 15)**. Melting curves confirmed amplification of single products in both the LPV3N **(Figure 16)** and actin qPCR reactions **(Figure 17)**, and agarose gel analysis of qPCR products confirmed the size of products as 77 bp **(Figure 18)**. Using standard curves constructed from known amounts of pure *P. cinnamomi* and avocado DNA **(Figure 19)**, pathogen load of each isolate in avocado stem material was determined **(Figure 20)**. Percentage *P. cinnamomi* DNA to total DNA ranged from 0.05% (FVB6T2S4) to 10.95% (942), but only 942 was present at significantly higher levels in stem material compared to other isolates.

Discussion

In this study, different components of pathogenicity, such as growth rate, sporulation and virulence were assessed in 12 *P. cinnamomi* isolates to gain a global understanding of the variability in virulence or fitness in *P. cinnamomi* isolates from avocado orchards in the Limpopo, Mpumalanga and KwaZulu-Natal provinces in South Africa and California, USA. Significant differences in *in vitro* growth rate, sporulation and virulence on avocado stems were observed between some isolates. The results of this study indicate phenotypic variability among *P. cinnamomi* isolates from avocado orchards, but no direct correlations were observed between different experiments or geographical origin of isolates. This study is the first to evaluate variation in phenotypical characters in *P. cinnamomi* isolates collected solely from avocado orchards in South Africa.

It is widely accepted that prolonged culturing of fungi and oomycetes has a negative effect on general fitness components such as growth, sporulation and virulence (NAKASONE *et al.* 2004). The negative effect of culture age on growth rate and pathogenicity has also been shown specifically for *P. cinnamomi* (LINDE *et al.* 1999b). In our study, by infecting apples with all 12 *P. cinnamomi* isolates, and re-isolating the pathogen after 10 days, we attempted to recover the virulence of the different isolates before growth, sporulation and virulence assays. Comparisons of growth rates, sporulation ability and virulence on avocado stems between isolates before and after plant infection needs to be performed. This will prove that re-isolating from plant material eliminates the negative effects associated with the long term maintenance of isolates in culture.

Studies have indicated that the optimal temperature for sporulation of *P. cinnamomi* is between 22°C and 28°C, with maximal sporulation at 26°C (CHEE and NEWHOOK 1964), thus sporulation assays were carried out at 25°C. Significant differences ($p<0.05$) in sporulation

were observed for some isolates, while a number of other isolates did not produce significantly different numbers of zoospores. Isolates FVB6T2S4, GKB4T9S4 and HB2T12R1 were identified as high sporulating isolates, and produced more than $4x10^4$ zoospores/ml, but zoospore concentrations were not significantly higher than what was observed for other isolates. TBB5T7S3 and HB8T12R1 were the poorest sporulaters, with zoospore concentrations of $2.16x10^4$/ml and $2.25x10^4$/ml obtained on average respectively. This was significantly lower than the average zoospore concentrations of FVB6T2S4, GKB4T9S4 and HB2T12R1. Zoospore production varied greatly between individual experiments, with the standard deviation for zoospore concentrations obtained in different repetitions and experiments ranging from 0.75 (HB8T12R1) to 3.7 (GKB4T9S4). This corresponded with the unreliable and inconsistent nature of *P. cinnamomi* sporangia production reported in literature (CHEE and NEWHOOK 1966; CHEN and ZENTMYER 1970; REITMANN 2013).

The growth rate, pathogenicity and reproduction of fungi and oomycetes are largely determined by temperature (ZENTMYER *et al.* 1976). Numerous studies in the 1960's indicated that *P. cinnamomi* grows optimally and with little response to temperature among isolates at temperatures between 20°C and 27°C. *Phytophthora cinnamomi* is considered a moderate-temperature pathogen, with minimum and maximum growth temperatures of below 10°C and above 33°C respectively (CHEE and NEWHOOK 1964; GALINDO and ZENTMEYER 1964; HASSIS *et al.* 1964). While these studies suggested that there was little variation in growth rates between *P. cinnamomi* isolates at a specified temperature on a specific nutritional media, studies conducted a decade later reported variation in both growth rates and cardinal temperatures (SHEPHERD and PRATT 1974; ZENTMYER *et al.* 1976), as well as mating-type specific responses to temperature (SHEPHERD *et al.* 1974).

Isolates were grown on 5% V8 agar, half strength PDA and MEA at 25°C, the optimal growth temperature of *P. cinnamomi,* for four days in order to determine whether there were any differences in growth rates between and within isolates on three different culture media. Significant differences ($p<0.05$) in growth rate was observed for different isolates on the same nutritional media, and individual isolates grew at different rates on 5% V8 agar, half strength PDA and MEA. Isolate HB2T12R1 had the highest growth rate on 5% V8 agar and PDA, and KB4T8R1 grew the slowest. On MEA, HB8T12R1 grew significantly faster than all other isolates, and 942 slower in comparison to other isolates. The results of this study correspond to what was observed in the studies conducted in the 1970's (SHEPHERD and PRATT 1974; SHEPHERD *et al.* 1974; ZENTMYER *et al.* 1976). The variation of isolates in response to different nutritional sources, as well as the differences in growth rate observed

for individual isolates on different culturing media, confirms the importance nutrition plays on growth rate (ZENTMYER *et al.* 1976), as reported in previous studies (CHEE and NEWHOOK 1964; HÜBERLI *et al.* 2001; LINDE *et al.* 1999b; ZENTMYER *et al.* 1976).

Inoculating stems of 2 year old Dusa® plants, which is regarded as a highly tolerant rootstock by Westfalia Technological Services, resulted in lesion formation for all isolates, indicating that all 12 isolates were pathogenic to avocado. The use of a tolerant avocado rootstock (Dusa®) allowed for discrimination between isolates based on virulence, which is not always possible when using a highly susceptible species (LINDE *et al.* 1999b). Significant differences in lesion lengths between some isolates indicated that variability with regard to virulence existed in the 12 isolates. The largest lesions were produced by HB5T11R2, GKB4T9S4 and FVB6T2S4 (in that order), and infection with TBB5T7S3, HB6T3S1 and 943 resulted in the smallest lesions. A number of previous studies have also shown significant differences in lesion formation between isolates (CHEE and NEWHOOK 1964; DUDZINSKI *et al.* 1993; HÜBERLI *et al.* 2001; LINDE *et al.* 1999b; ROBIN and DESPREZ-LOUSTAU 1998). The site of inoculation could explain differences in lesion length data, and subsequent large standard deviations for some isolates. Each plant was inoculated twice, and in some cases the site of inoculation was on thinner branches if a second inoculation site was not available on the thicker stem. Differences in defence responses in stems or less mature branches could have resulted in the differences in lesion lengths for individual isolates.

As stem inoculations only assess the ability of a pathogen to cause disease once it is in its host, stem inoculations are not the preferred method for assessing variation in virulence in root pathogens such as *P. cinnamomi*. However, the small numbers of zoospores produced by some isolates made the production of adequate and equal amounts of zoospores for root inoculation impossible. Although *P. cinnamomi* is a root pathogen, previous studies have indicated that stem or trunk inoculations can be used to assess variability in host responses to *P. cinnamomi* (DIXON and THINLAY 1984; MARKS *et al.* 1981). Once a standardized set of isolates has been selected for rootstock field trials, dip-inoculation in a zoospore suspension or potting mix inoculation, which mimics the natural infection process, should be used (ROBIN and DESPREZ-LOUSTAU 1998).

Pathogen quantification in diseased stem material produced unexpected results. Isolate 942 was the only isolate present at significant higher levels in avocado stem material compared to the other isolates. Isolate 942 showed average lesion lengths, and consequently results of quantification of disease load were unexpected. Infection with HB5T11R2 and GKB4T9S4 resulted in the development of lesions that were significantly larger than those produced by

other isolates. It was expected that the amount of *P. cinnamomi* DNA present in plant material infected with these two isolates would be higher as a result of the larger lesions. A plausible explanation for the mentioned results is that 942 was a superior colonizer, capable of destroying plant tissue more effectively than other isolates. This experiment should be repeated to determine whether higher pathogen load for isolate 942 was due to increased colonization ability, or chance events and technical error.

No single isolate consistently performed the best across the four different experiments, and geographic origin of isolates did not appear to play a role in pathogenicity, as observed in previous studies in which no correlations between variation in different phenotypes, or interactions with geographic origin was observed (HÜBERLI *et al.* 2001; LINDE *et al.* 1999b). KB13T4R3 was amongst the top growers on all three nutritional media, while KB4T8R1 was consistently one of the worst. Some isolates sporulated well and were more virulent on avocado stems, while others which grew and sporulated well displayed an average level of virulence on avocado. Isolates FVB6T2S4 and GKB4T9S4, which were the two highest sporulaters, ranked in the top three with regard to its virulence on avocado, but FVB6T2S4 was one of the slowest growers on both V8 agar and PDA. HB2T12R1, another high sporulating isolate, was the fastest growing isolate on both V8 agar and PDA, but displayed average virulence on avocado stems. TBB5T7S3 produced the least number of zoospores, and also caused the smallest lesion on avocado stems, but had the second highest growth rate on V8 agar. Another poor sporulator, HB8T12R1 had the biggest colony diameter on MEA.

In the most recent study to evaluate differences in pathogenicity in *P. cinnamomi* isolates in South Africa, a positive correlation was found between *in vitro* growth rate and pathogenicity in the field (LINDE *et al.* 1999b). This prompted the authors to suggest that fast growing isolates can be used to screen plant material for susceptibility, tolerance or resistance (LINDE *et al.* 1999b). That relationship was, however, not linear, and in conjunction with the results of this study, challenge with a mixed inoculum might be a better approach. Mixed inoculation with FVB6T2S4, GKB4T9S4, HB2T12R1, HB5T11R2 and 942 can be used to screen avocado plant material for resistance in future, as this subset of isolates includes isolates that sporulate and grow well, and which are also aggressive on avocado in terms of lesion length and colonization ability.

Some of the earliest studies on *P. cinnamomi* suggested that specific pathogenicity, the behaviour of an isolate on a specific host, differed among isolates of *P. cinnamomi*, with some isolates able to infect a specific host, while others could not (ZENTMYER and

GUILLEMET 1981). Some authors suggested that different races, strains or “biotypes” of *P. cinnamomi* existed (MANNING and CROSSAN 1996; ZENTMYER and GUILLEMET 1981). However, in more recent studies it has been shown that variation among *P. cinnamomi* isolates should rather be attributed to virulence, as different isolates can all infect different host species, with only the severity of the infection differing between isolates (ROBIN and DESPREZ-LOUSTAU 1998). In future, this study should be expanded to include a larger number of isolates, and the addition of mating type and genotypic data would contribute to the selection of highly pathogenic, well-characterized isolates for rootstock screening trials, as both phenotypic and genotypic variation could have an effect on pathogenicity (LINDE *et al.* 1999b). This study successfully characterized a set of *P. cinnamomi* isolates in terms of phenotypic variability for use in infection studies. This will ensure that rootstocks are challenged by a group of isolates representative of the most virulent isolates during infection trials.

Conclusion

Variation in phenotypical traits such as growth rate, sporulation and most importantly, virulence to avocado in *P. cinnamomi* isolates has important applications in both basic and applied research. Selection of isolates used to screen promising new rootstock varieties by industry partners Westfalia, as well as isolates used in research should be carefully considered. The robustness of resistance is dependent on the amount of variation in pathogen populations, highlighting the importance of using a mixed inoculum, representative of the variation present in the whole population, in screening and infection trials. This study confirmed the presence of significant variation in traits associated with pathogenicity in *P. cinnamomi* isolates from avocado orchards. A subset of isolates, representing isolates that grow and sporulate well, as well as isolates with high levels of virulence to avocado were identified for use in future research.

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Tables

Table 1. *Phytophthora cinnamomi* isolates used in this study.

Isolate	Origin	Source of isolation
GKB4T9S4	Tzaneen, Limpopo Province, SA	Soil
TBB5T7S3	Tzaneen, Limpopo Province, SA	Soil
KB13T4R3	Howick, KwaZulu-Natal, SA	Roots
942	Riverside, California, USA	Trunk
FVB6T2S4	Tzaneen, Limpopo Province, SA	Soil
HB8T12R1	Hazyview, Mpumalanga, SA	Roots
943	Riverside, California, USA	Trunk
KB4T8R1	Howick, KwaZulu-Natal, SA	Roots
HB2T12R1	Hazyview, Mpumalanga, SA	Roots
941	Riverside, California, USA	Trunk
HB5T11R2	Hazyview, Mpumalanga, SA	Roots
HB6T3S1	Hazyview, Mpumalanga, SA	Soil

Table 2. Polymerase chain reaction (PCR) primers used for *Phytophthora cinnamomi* quantification in *Persea americana* stems.

Primer	Primer sequence (5'-3')	Target gene	Product size (bp)	Reference
LPV3-sense	GTCCAGACTGTCGATGTG	*Lpv3*	450	Kong *et al.* (2003)
LPV3-antisense	GAACCACAACAGGCACGT	*Lpv3*		Kong *et al.* (2003)
LPV3N-sense	GTCACGACCATGTTGTTG	*Lpv3*	77	Engelbrecht *et al*. (2013)
LPV3N-antisense	GAGGTGAAGGCTGTTGAG	*Lpv3*		Engelbrecht *et al*. (2013)
Actin-sense	GTATTCATTCACCACTACTG	*Actin*	77	Engelbrecht *et al*. (2013)
Actin-antisense	AGTCAAGAGCCACATAAG	*Actin*		Engelbrecht *et al*. (2013)

Figures

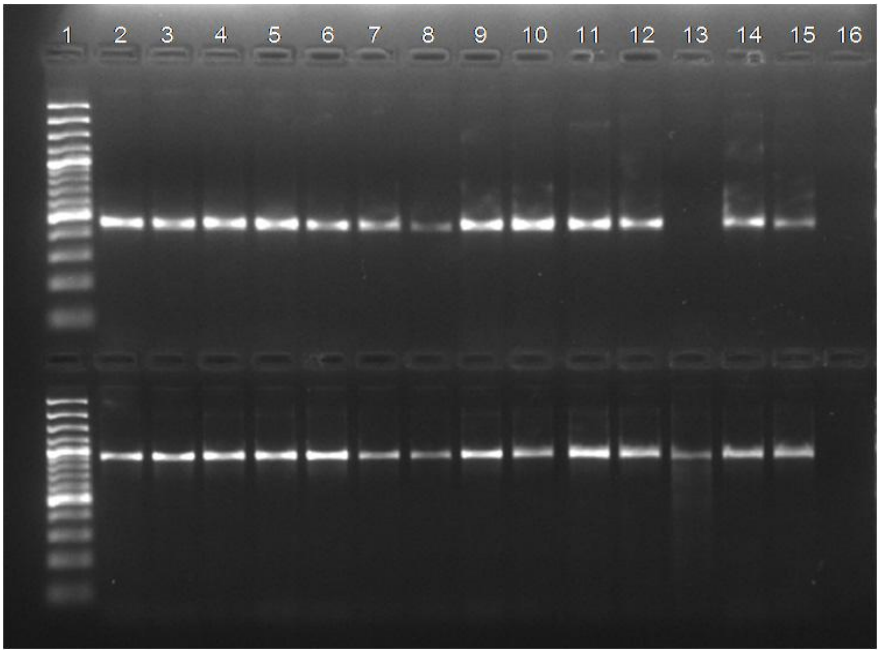

Figure 1. **Agarose gel electrophoresis (2%, 80 V) of PCR products produced by the LPV3 primer pair (top), and PCR products produced by the ITS 4 and ITS 6 primer pair (bottom) for identification of isolates as *Phytophthora cinnamomi*.** Lane 1: 100 bp molecular weight standard (O'Generuler, Thermo Scientific); 2: HB8T12R1; 3: KB4T8R1; 4: FVB6T2S4; 5: 942; 6: HB6T3S1; 7: HB5T11R2; 8: 943; 9: GKB4T9S4; 10: 941; 11: KB13T4R3; 12: HB2T12R1; 13: 435; 14: TBB5T7S3; 15: positive control (*P. cinnamomi*); 16: negative (water) control.

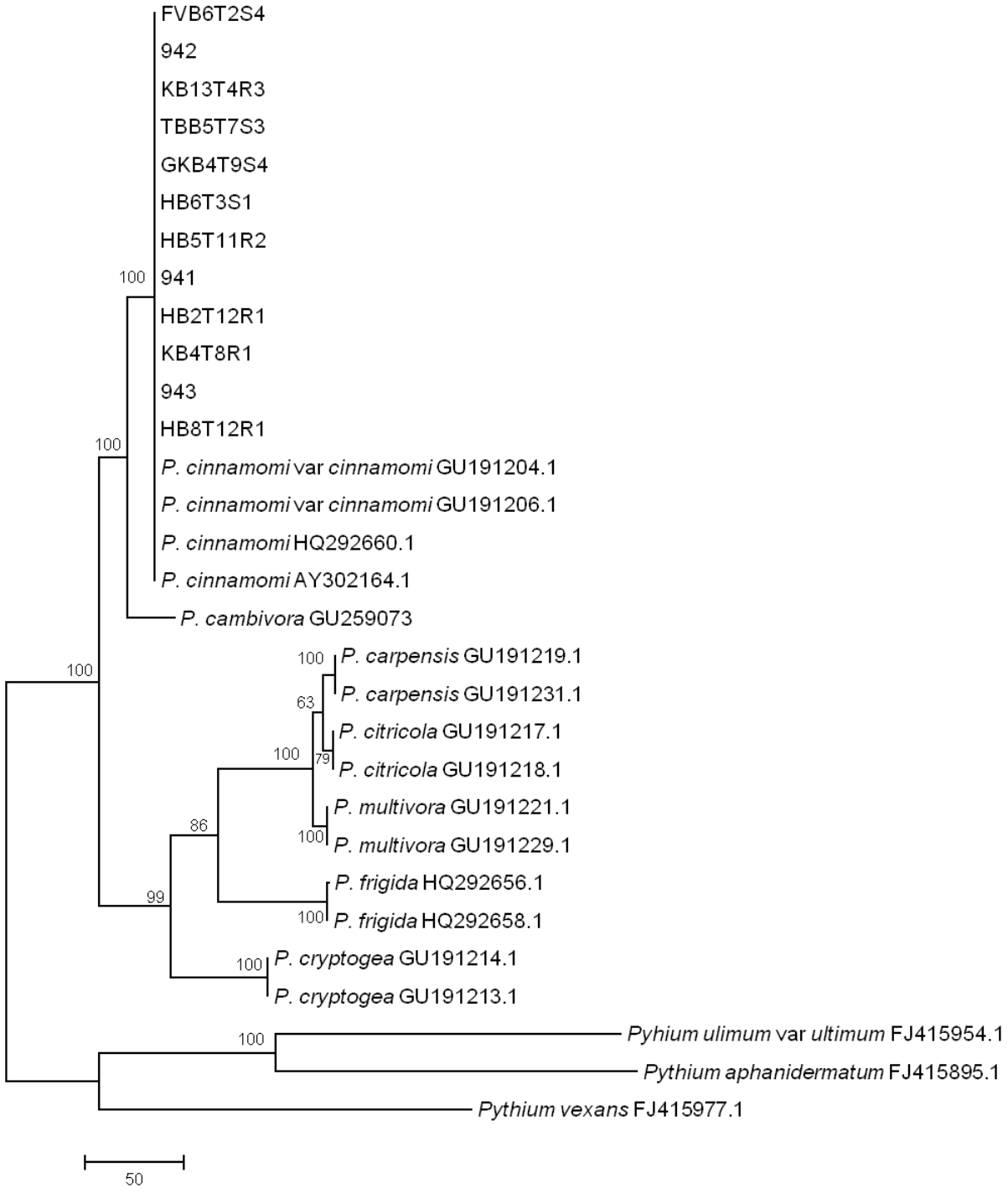

Figure 2. Phylogenetic tree generated by parsimony analysis of ITS sequence data from *Phytophthora* spp. Branch support is indicated by bootstrap values above branches, and accession numbers of sequence data in NCBI is given. *Pythium ultimum* var *ultimum*, *Pythium vexans* and *Pythium aphanidermatum* were used as outgroups.

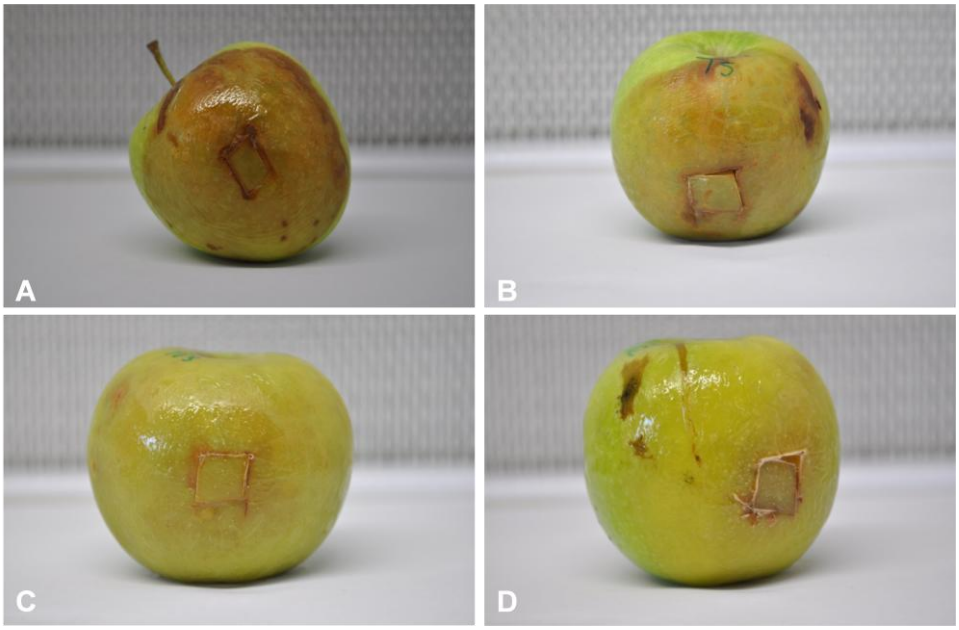

Figure 3. Disease symptoms on Granny Smith apples inoculated with *Phytophthora cinnamomi*. Some isolates caused severe disease symptoms (A: KB4T8R1 and B: FVB6T2S4), while apples inoculated with other isolates appeared healthy (C: GKB4T9S4 and D: KB13T4R3).

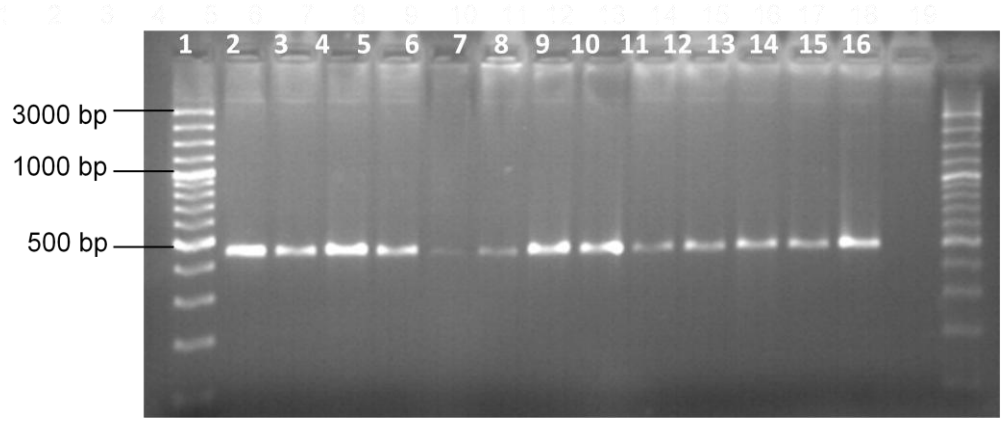

Figure 4. Agarose gel electrophoresis (2%, 80V) of PCR products produced by the LPV3 primer pair confirming the successful reisolation of *Phytophthora cinnamomi* from diseased apple material. Lane 1: 100 bp molecular weight standard (O'Generuler, Thermo Scientific); 2: HB2T12R1; 3: TBB5T7S3; 4: 941; 5: 942; 6: 943; 7: KB13T4R3; 8: HB6T3S1; 9: HB5T11R2; 10: KB4T8R1; 11: HB8T12R1; 12: FVB6T2S4; 13: GKB4T9S4; 14: positive control; 15: negative (water) control; 16: 100 bp molecular weight standard (O'Generuler, Thermo Scientific).

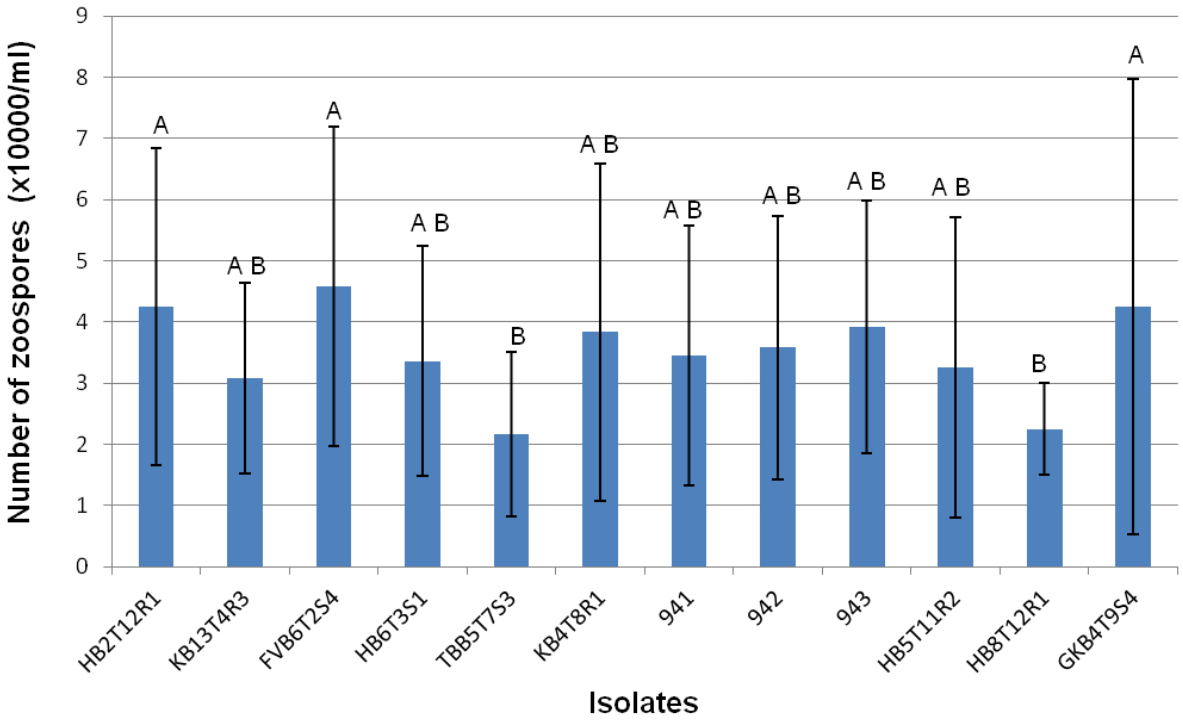

Figure 5. Sporulation ability of *Phytophthora cinnamomi* isolates. Sporulating mycelia were produced *in vitro*, and zoospores released by cold shock. Error bars for each data point represents standard deviation. Statistical analysis was performed using analysis of variance (ANOVA) and a Least Square Means Differences Students' t-test at a significance level of $p<0.05$ (JMP '9 software, SAS, North Carolina, USA). Bars containing the same letters did not show significant differences at a confidence interval of $p<0.05$. Data represents three technical repeats of an experiment repeated three times.

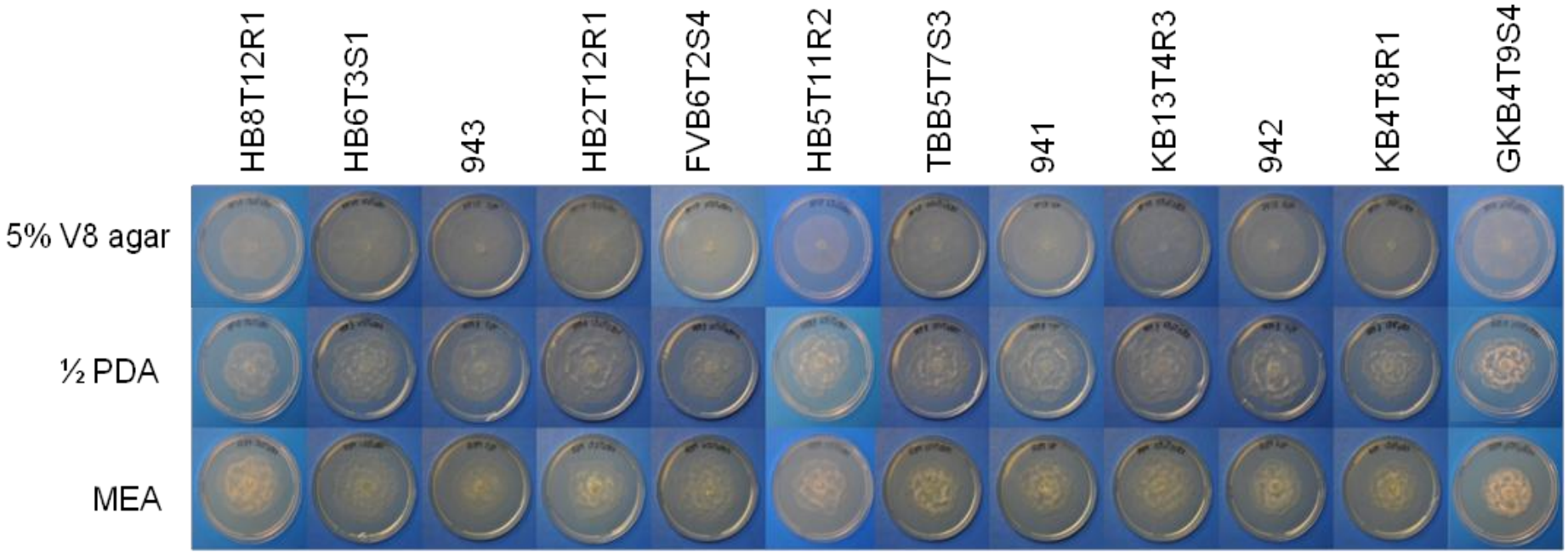

Figure 6. Culture morphology of *Phytophthora cinnamomi* on three different culture media. All isolates grew in a white, radial pattern on 5% V8 agar, and showed the characteristic rosette or scalloped shape on half strength PDA and MEA.

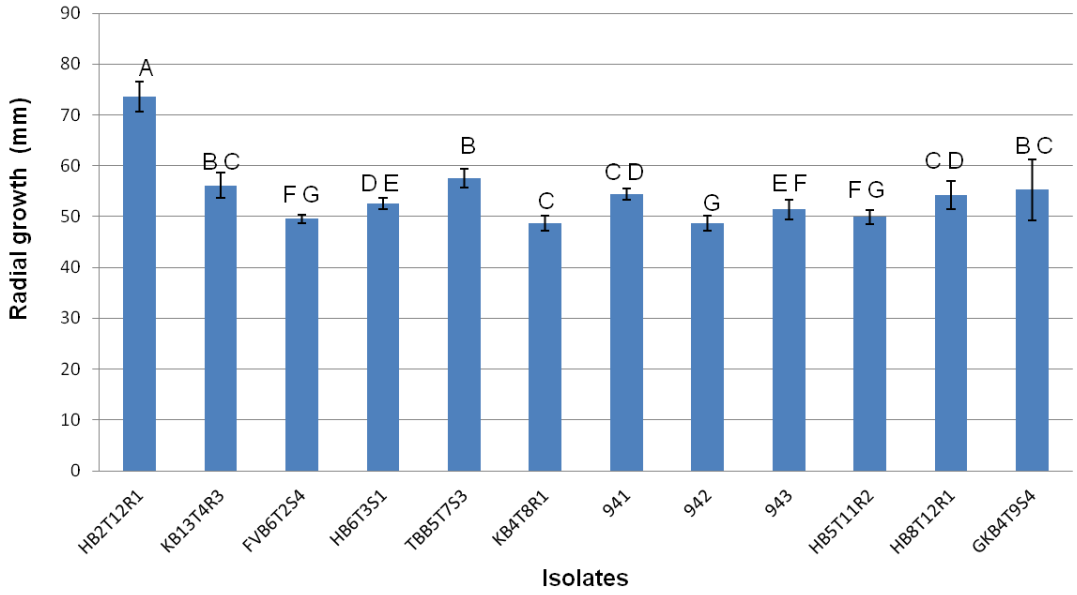

Figure 7. Radial growth of *Phytophthora cinnamomi* isolates after 4 days on 5% V8 agar . Error bars for each data point represents standard deviation. Statistical analysis was performed using analysis of variance (ANOVA) and a Least Square Means Differences Students' t-test at a significance level of $p < 0.05$ (JMP '9 software, SAS, North Carolina, USA). Bars containing the same letters did not show significant differences at a confidence interval of $p<0.05$. Data represents the average of five technical repeats.

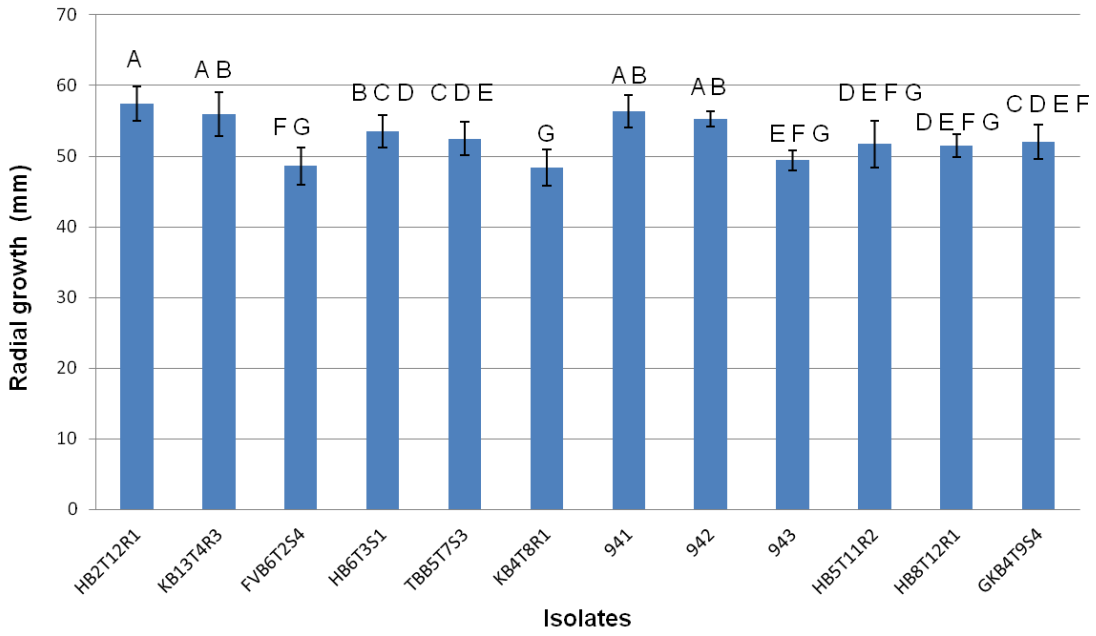

Figure 8. Radial growth of *Phytophthora cinnamomi* isolates after 4 days on half strength PDA. Error bars for each data point represents standard deviation. Statistical analysis was performed using analysis of variance (ANOVA) and a Least Square Means Differences Students' t-test at a significance level of $p<0.05$ (JMP '9 software, SAS, North Carolina, USA). Bars containing the same letters did not show significant differences at a confidence interval of $p<0.05$. Data represents the average of five technical repeats.

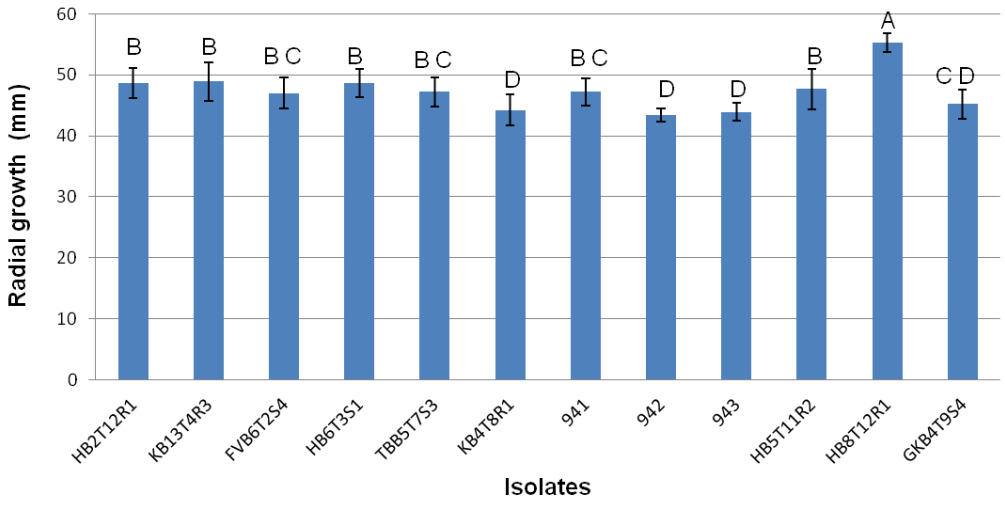

Figure 9. Radial growth of *Phytophthora cinnamomi* isolates after 4 days on MEA. Error bars for each data point represents standard deviation. Statistical analysis was performed using analysis of variance (ANOVA) and a Least Square Means Differences Students' t-test at a significance level of $p< 0.05$ (JMP '9 software, SAS, North Carolina, USA). Bars containing the same letters did not show significant differences at a confidence interval of $p<0.05$. Data represents the average of five technical repeats.

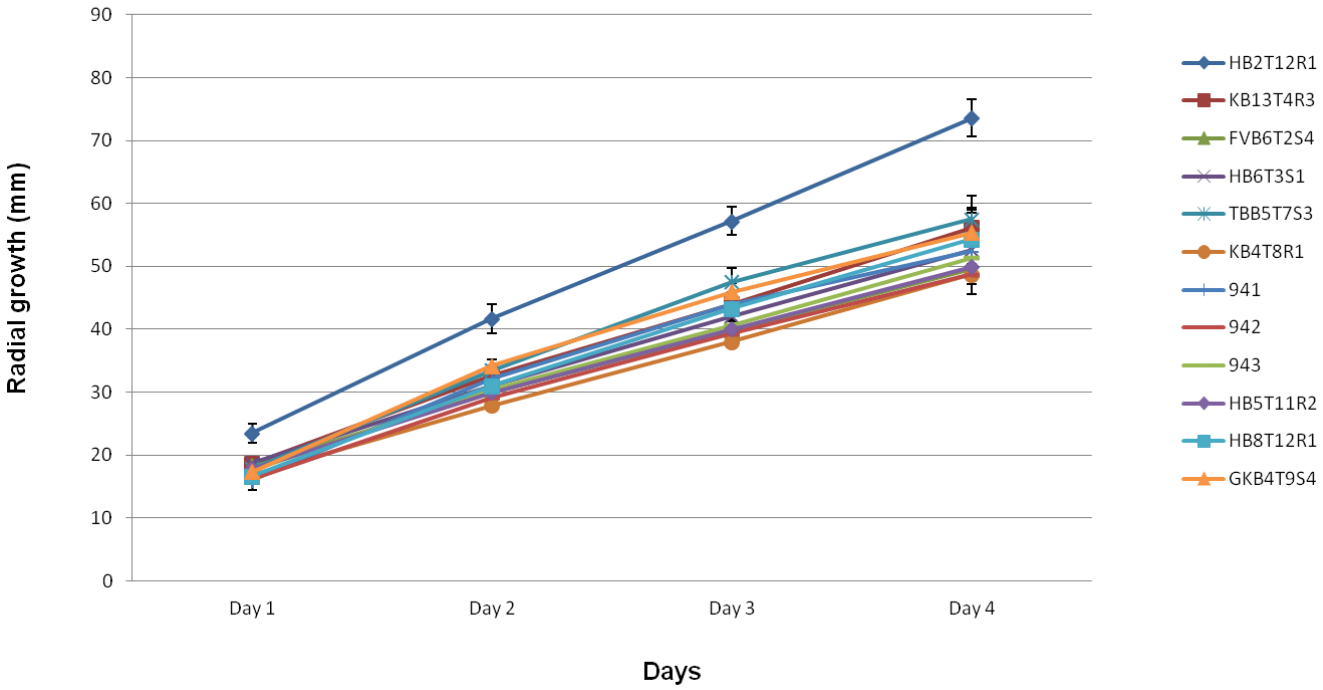

Figure 10. Growth rate of *Phytophthora cinnamomi* isolates on 5% V8 agar over four days. Error bars indicate standard deviation, and is indicated for each data point.

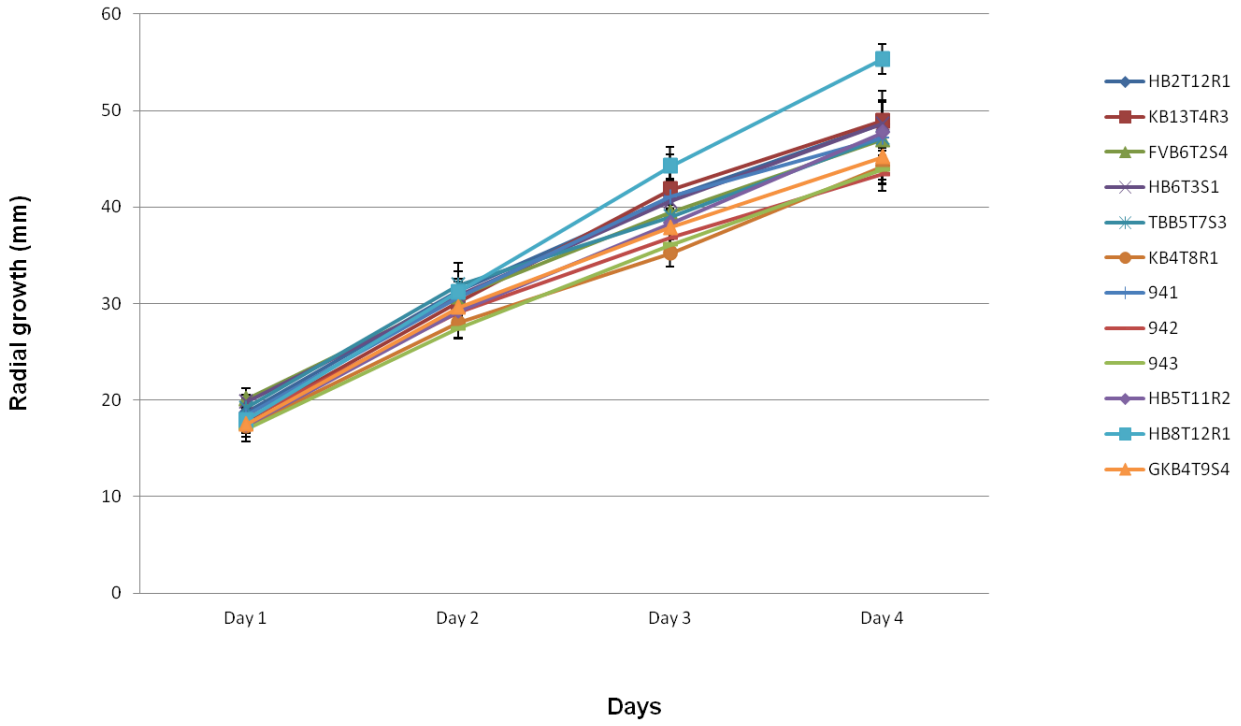

Figure 11. Growth rate of *Phytophthora cinnamomi* isolates on MEA over four days. Error bars indicate standard deviation, and is indicated for each data point.

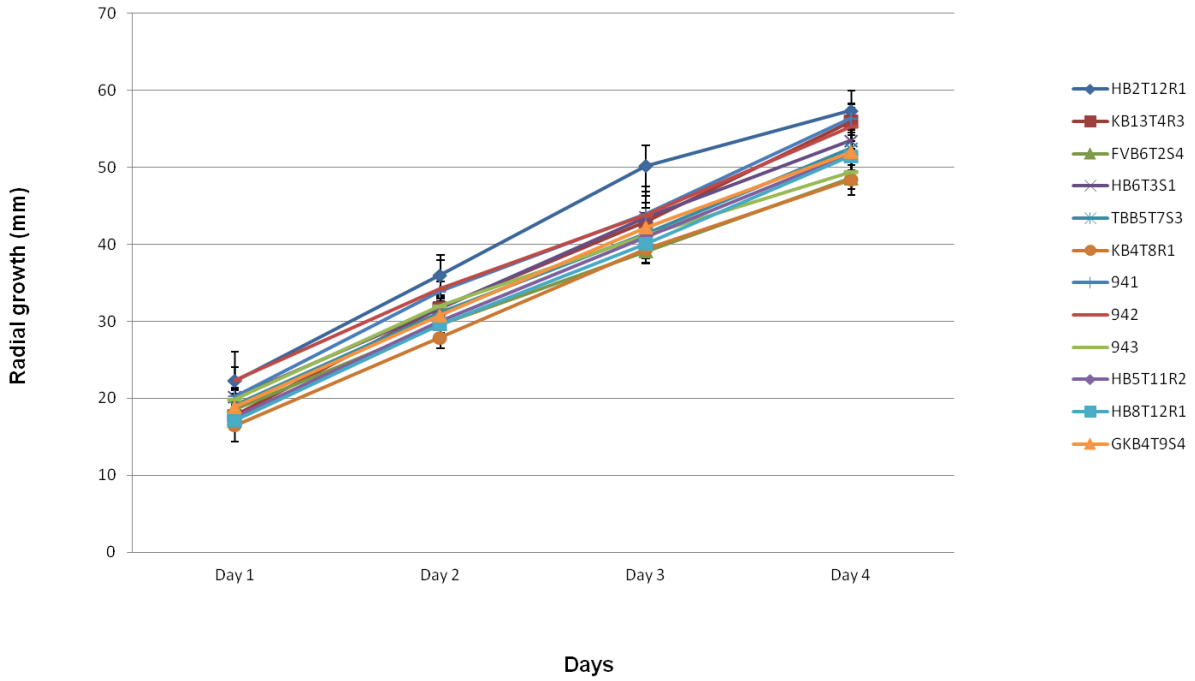

Figure 12. Growth rate of *Phytophthora cinnamomi* isolates on half strength PDA over four days. Error bars indicate standard deviation, and is indicated for each data point

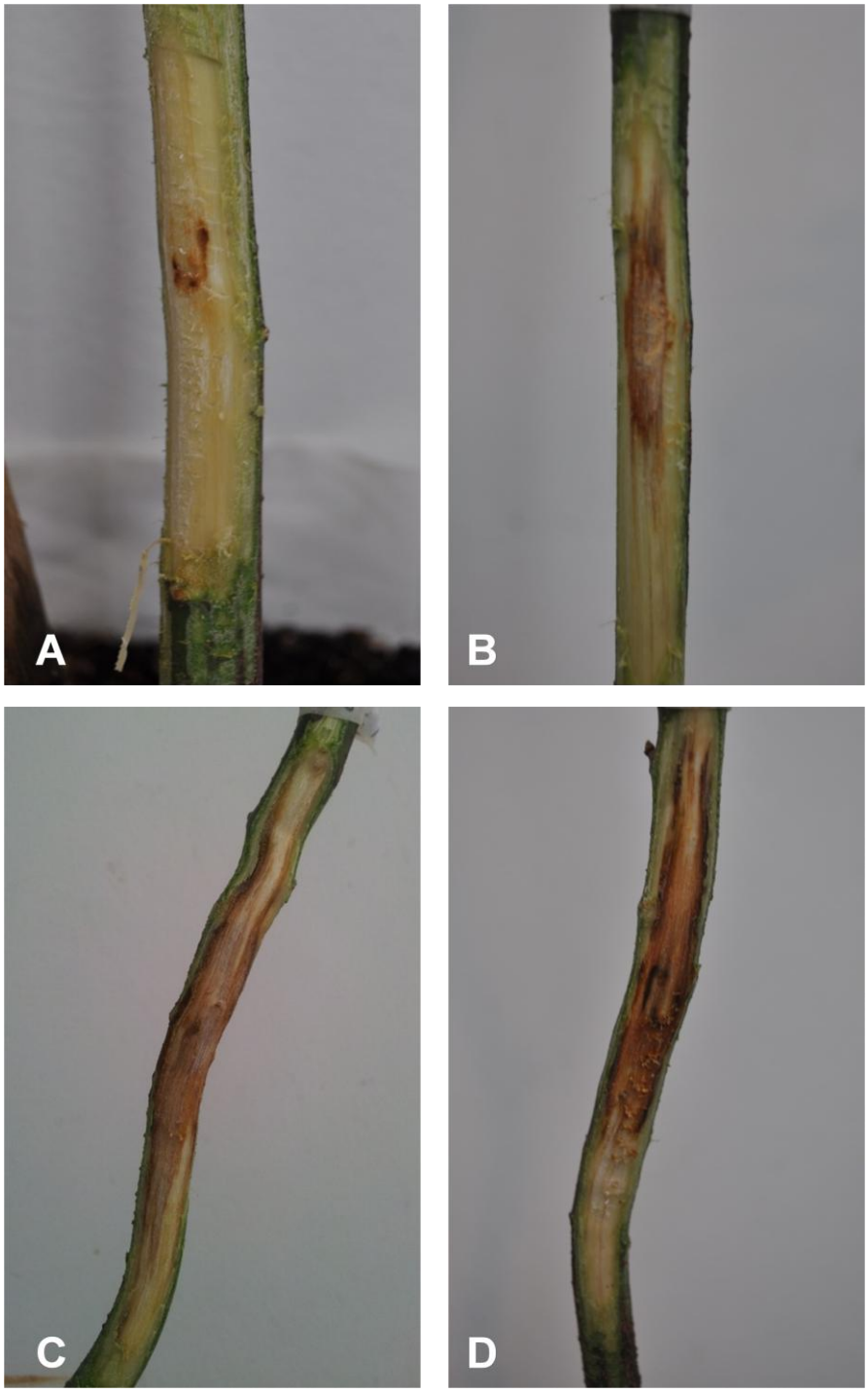

Figure 13. Lesions formed after five weeks following inoculation of two year old Dusa® avocado trees with *Phytophthora cinnamomi.* A: Control (inoculated with sterile 5% V8 agar; B: TBB5T7S3; C: HB5T11R2; D: HB2T12R1.

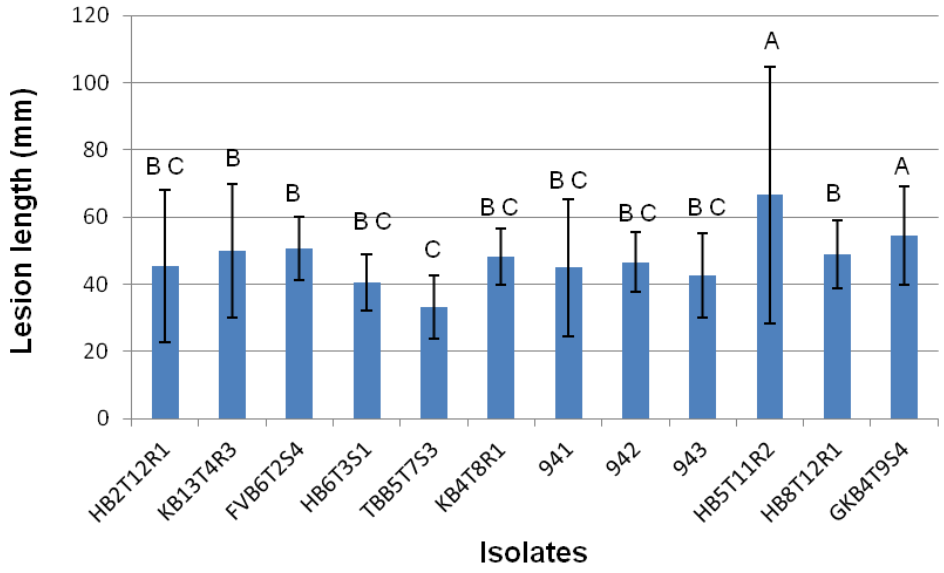

Figure 14. Average lesion lengths measured five weeks after inoculation of avocado stems with *Phytophthora cinnamomi*. Error bars for each data point represents standard deviation. Statistical analysis was performed using analysis of variance (ANOVA) and a Least Square Means Differences Students' t-test at a significance level of $p<0.05$ (JMP '9 software, SAS, North Carolina, USA). Bars containing the same letters did not show significant differences at a confidence interval of $p<0.05$.

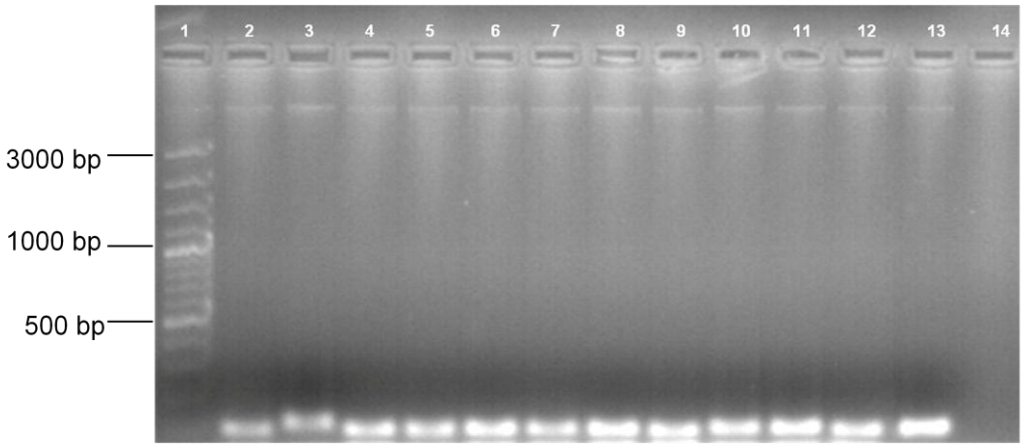

Figure 15. Agarose gel electrophoresis (1%, 80V) of PCR products produced by the LPV3N primer pair confirming successful infection of avocado stem material with *Phytophthora cinnamomi*. Lane 1: 100 bp molecular weight standard (O'Generuler, Thermo Scientific); 2: HB8T12R1; 3: KB4T8R1; 4: HB2T12R1; 5: KB13T4R3; 6: 941; 7: 942; 8: HB5T12R1; 9: FVB6T2S4; 10: TBB5T7S3; 11: GKB4T9S4; 12: 943; 13: HB6T3S1; 14: negative (water) control.

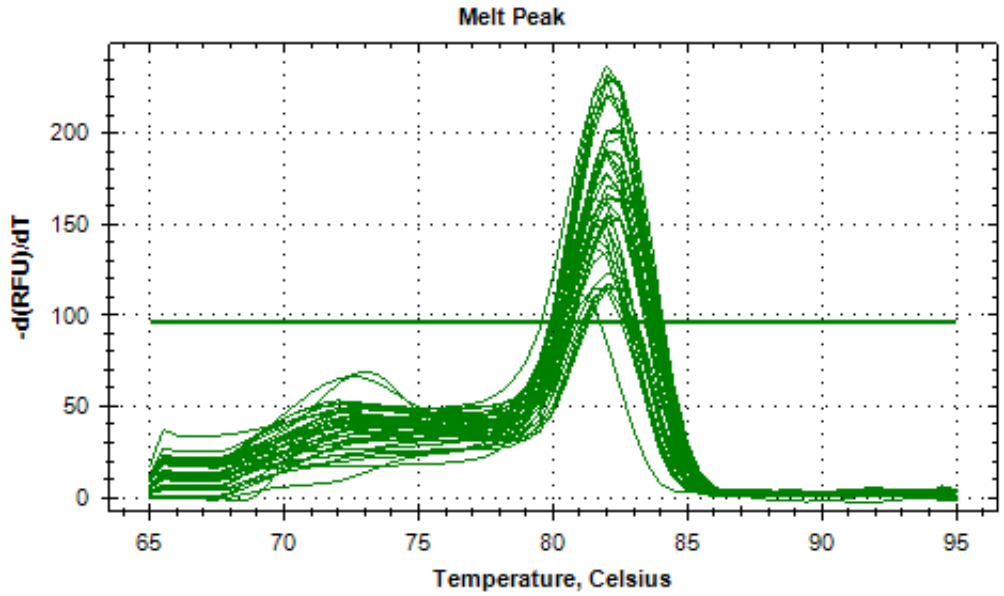

Figure 16. Melting curve analysis of LPV3N real-time qPCR samples. Melting temperature (Tm) of the LPV3N amplicons was determined by plotting the negative first derivative of the normalized fluorescence against temperature.

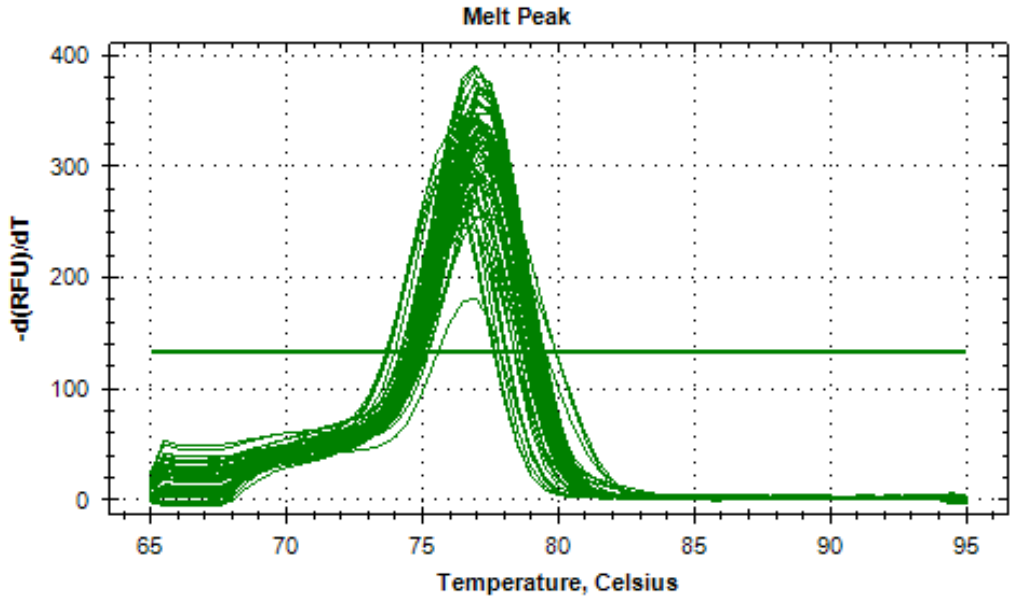

Figure 17. Melting curve analysis of actin real-time qPCR samples. Melting temperature (Tm) of the actin amplicons was determined by plotting the negative first derivative of the normalized fluorescence against temperature.

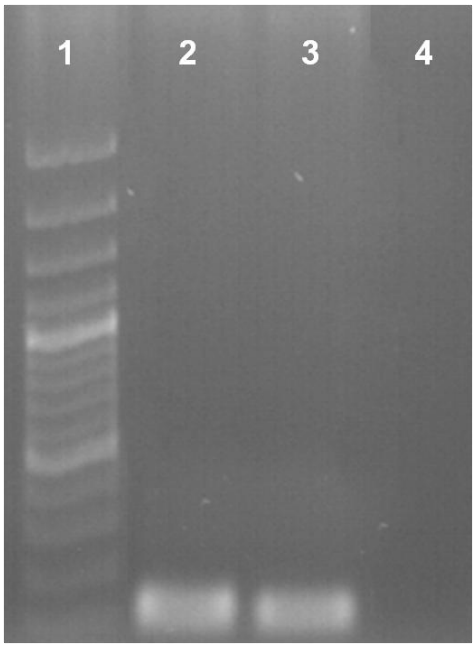

Figure 18. **Agarose gel electrophoresis (1%, 80 V) of PCR products produced by the LPV3N and actin primer pairs.** 1: 100 bp molecular weight standard (O'Generuler); 2: 77 bp LPV3N PCR product; 3: 77 bp actin PCR product; 4: negative (water) control.

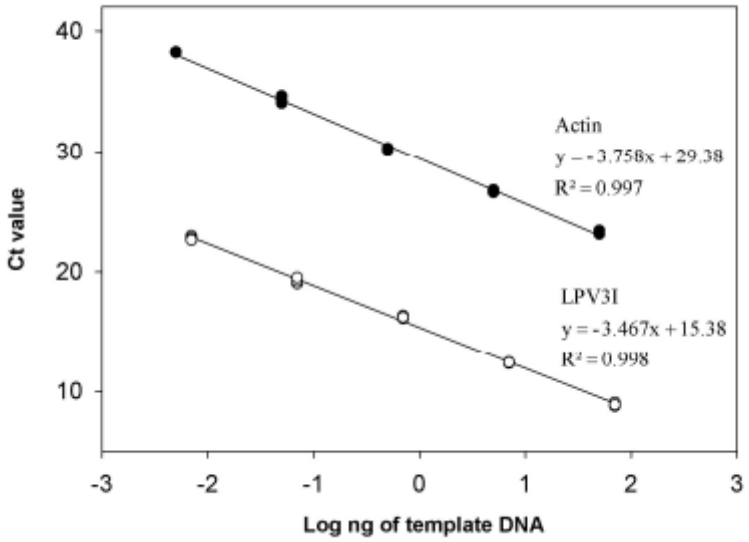

Figure 19. Standard regression curves of *Phytophthora cinnamomi* and avocado DNA used for pathogen quantification. A dilution series (1:0; 1: 10; 1:100; 1:1000; and 1:10000) of *P. cinnamomi* and avocado DNA used as template for PCR amplification of the *actin* (actin-sense and actin-antisense) and *Lpv* (LPV3N-sense and LPV3N-antisense) genes was used to generate standard curves for each primer pair. Image obtained from Engelbrecht *et al.* (2013).

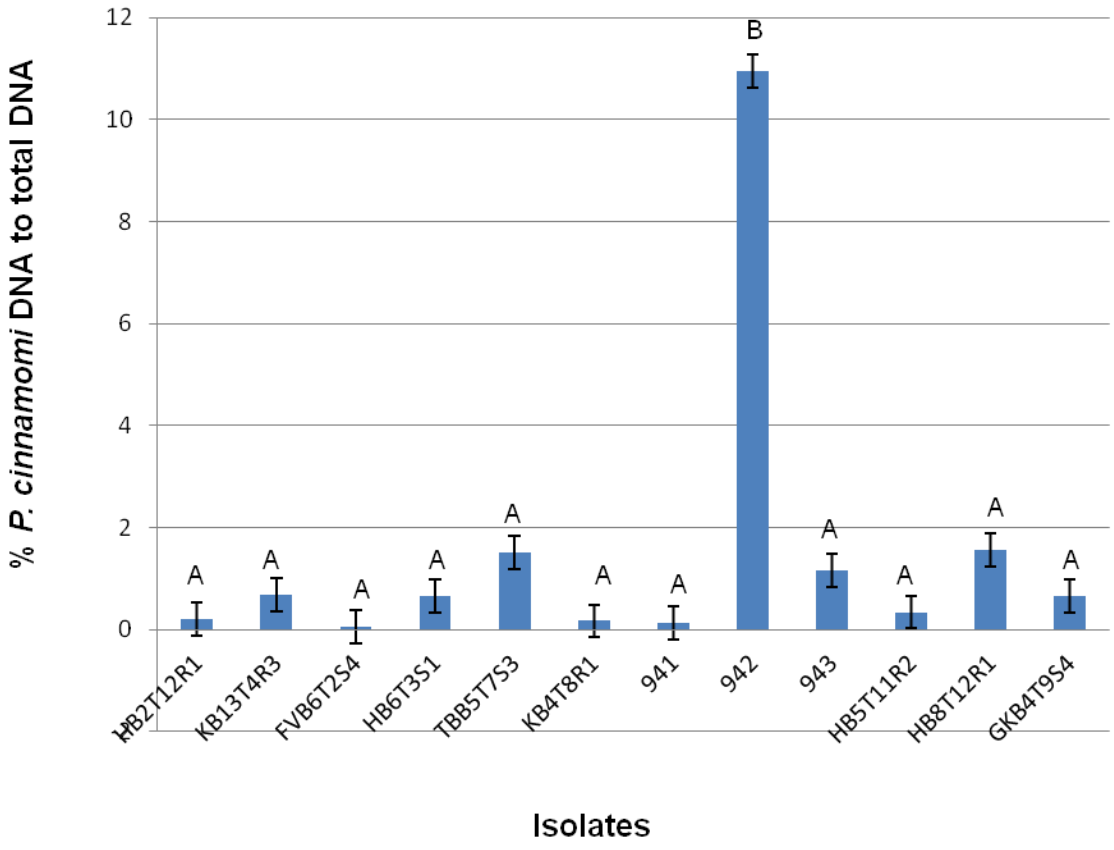

Figure 20. Quantification of *Phytophthora cinnamomi* DNA in DNA extracted from avocado stems. Error bars for each data point represents standard deviation. Statistical analysis was performed using analysis of variance (ANOVA) and a Least Square Means Differences Students' t-test at a significance level of $p<0.05$ (JMP '9 software, SAS, North Carolina, USA). Bars containing the same letters did not show significant differences at a confidence interval of $p<0.05$.

Chapter 4

General Discussion

Phytophthora cinnamomi Rands is one of the most well-known species in the genus *Phytophthora* due to its extremely large host range, which includes dozens of important crop species as well as native vegetation such as the indigenous fynbos in South Africa and *Eucalyptus marginata* in Australia (HARDHAM 2005; VON BROEMBSEN 1984). As a result, *P. cinnamomi* infection results in significant losses in agriculture annually, and also poses a serious threat to natural ecosystems and biodiversity (CAHILL *et al.* 2008; PODGER *et al.* 1965; VON BROEMBSEN 1984). In South Africa, *P. cinnamomi* is of particular importance to the avocado (*Persea americana* Mill.) industry, as *P. cinnamomi* infection leads to the development of Phytophthora root rot (PRR) in susceptible trees. PRR results in necrosis of the fine feeder roots, leading to tree dieback and death, which reduces yields of this economically important export fruit crop.

Currently, control of PRR in avocado orchards is achieved through a combination of chemical control agents, PRR-tolerant rootstocks and good management practices. Phosphite trunk injections are successfully employed as a means of chemical control (GUEST and GRANT 1991; HARDY and BARRET 2001), but the evolution of pathogen genotypes resistant to chemical control agents always remain a threat (DOBROWOLSKI *et al.* 2008). Due to more recent shifts to organic agriculture to limit the negative effects of pesticides on the environment, avocado rootstocks highly tolerant or fully resistant to infection by *P. cinnamomi* are required for continued sustainable and industrious avocado production (LI *et al.* 2013). Specific plant mechanisms associated with tolerance or resistance in rootstocks have not been identified, hindering the production of superior rootstocks in the short term.

An improved understanding of defence responses activated in *P. americana* upon attack by *P. cinnamomi* will aid in identifying the mechanisms which allow tolerant rootstocks to limit or restrict damage caused by pathogen attack. The first aim of this study was to tag *P. cinnamomi* with a fluorescent protein gene in order to study the interaction between avocado and *P. cinnamomi in planta*, with a special interest in the events associated with the switch from biotrophy to necrotrophy. The large number of *Phytophthora* spp. previously transformed using PEG and $CaCl_2$ mediated protoplast transformation led to the selection of this technique for the production of fluorescently-tagged *P. cinnamomi* isolates (AH-FONG and JUDELSON 2011; BOTTIN *et al.* 1999; CHEN *et al.* 2009; DUNN *et al.* 2013; HORTA *et al.* 2008; LE BERRE *et al.* 2008b; MCLEOD *et al.* 2008; SI-AMMOUR *et al.* 2003).

Phytophthora cinnamomi US isolates 941, 942, 943 and 2060 were compared for their sporulation ability in order to select an isolate for transformation. *In vitro* production of

zoospores of *P. cinnamomi* is notoriously difficult and inconsistent (CHEN and ZENTMYER 1970), and a high sporulating isolate was required to enable production of young germlings. Two protocols previously used for protoplasting and transformation of *Phytophthora infestans*, *Phytophthora sojae*, *Phytophthora citricola*, *Phytophthora aphanidermatum* and many other *Phytophthora* spp. were evaluated for releasing protoplasts from mycelia and young germlings of US *P. cinnamomi* isolate 943 (JUDELSON *et al.* 1991; MCLEOD *et al.* 2008). The method described by Judelson *et al*. (1991) resulted in higher yields of protoplasts for one- and two-day-old mycelia, as well as young germlings, and variations of this method have been used widely to transform other *Phytophthora* spp (JUDELSON *et al.* 1991).

This experiment described above was followed by evaluation of different mannitol concentrations in 5% V8 broth for optimal rates of protoplast regeneration. Mannitol acts as an osmoticum when regenerating the cell walls of protoplasts, and the highest regeneration rates were observed at 0.7 M mannitol. Based on this study and what has been reported in literature (HORTA *et al.* 2008; JUDELSON and MICHELMORE 1991; JUDELSON *et al.* 1991; MCLEOD *et al.* 2008), protoplasts will regenerate at a range of mannitol concentrations (0.5 M-1 M). However, at higher concentrations, regeneration rates are lower, and at lower concentrations germtubes grow too fast, preventing isolation of single transformant colonies.

After failing to obtain transformants during a research visit to the US, a South African isolate, TBB5T7S3, which was used for transcriptome sequencing of *P. cinnamomi,* was selected for transformation in order to compensate for possible isolate-specific effects on transformation. Despite the fact that using young germlings resulted in higher protoplast yields in previous experiments, mycelia was used as starting material for protoplasting, as TBB5T7S3 sporulated poorly. Both the methods of Judelson *et al.* (1991) and McLeod *et al.* (2008) for protoplasting and transformation were used as protoplast yield did not differ greatly when digesting older cultures. Two putative GFP transformants were identified based on their growth on selection media and the presence of the *GFP* gene, but no fluorescence was detected using the DFP-1™ Dual Fluorescent Protein Flashlight and fluorescence microscopy.

The lack of GFP protein expression could be due to integration of the plasmid DNA into regions of the genome which are not transcriptionally active or into regions which induce methylation and silencing of transgenes (JUDELSON and AH-FONG 2009; KOOTER *et al.* 1999; PRÖLS and MEYER 1992). Instability and spontaneous loss of introduced genes have been reported in *Phytophthora parasitica*, and the coenocytic nature of the mycelia of some

Phytophthora spp., which can result in the formation of heterokaryons following transformation, was cited as a possible way by which this could have occurred (GAULIN *et al.* 2007).

Integration of plasmid DNA into hererochromatic, repeat-rich regions of the genome, such as regions near telomeres and centromeres will prevent gene expression due to chromatin condensation which prevents access for transcription machinery (PRÖLS and MEYER 1992). Loss of transgenes is also more common in transformation of oomycetes, as foreign genes are rarely introduced through homologous recombination, which is used with great success in fungi (RUIZ-DÍEZ 2002). The development of vectors enabling integration of foreign DNA by homologous recombination might improve oomycete transformation efficiencies, and may also contribute to targeted gene manipulations through mechanisms other than gene silencing.

The inconsistency associated with protoplast production, and low protoplast regeneration efficiencies contribute to low transformation efficiencies in *P. cinnamomi* and other oomycetes (MCLEOD *et al.* 2008). Transformation of *P. cinnamomi* has only been reported twice in literature (BAILEY *et al.* 1993; HORTA *et al.* 2008), in contrast to model species such as *P. infestans* and *P. sojae,* for which dozens of transformation studies have been published. The small pool of available literature for *P. cinnamomi* transformation is indicative of, and a contributing factor to, the difficulty of transforming *P. cinnamomi*. The ability to consistently release larger numbers of good quality protoplasts, coupled with increased protoplast regeneration rates will contribute towards achieving higher levels of stable transformation (MCLEOD *et al.* 2008). Alternative vectors, which are better able to drive expression of selectable marker genes can be considered to make selection of stable transformants more easy in future (MCLEOD *et al.* 2008). A biolistics approach, which has been successfully used in *P. cinnamomi* and other *Phytophthora* spp., can also be considered, as well as *Agrobacterium*-mediated transformation, which has been successfully applied in *P. infestans* (VIJN and GOVERS 2003).

The recent identification of more than 2000 putative pathogenicity genes for *P. cinnamomi* stresses the importance of the establishment of a transformation procedure for *P. cinnamomi* to allow for functional validation of these genes (REITMANN 2013). Pathogenicity factors identified included RXLR-effectors, crinkling and necrosis-inducing factors and cell wall degrading enzymes, but of special interest to the Fruit Tree Biotechnology Programme are the 46 putative RXLR effectors that were identified. Silencing of these genes in *P.*

cinnamomi by transformation with anti-sense silencing constructs will aid our understanding of how *P. cinnamomi* manipulates host cells.

The second aim of this study was to re-evaluate the amount of phenotypic variation present in South African *P. cinnamomi* isolates, focussing on isolates collected from avocado orchards. Previous studies had indicated that significant levels of phenotypic variation were present in clonal South African *P. cinnamomi* populations (LINDE *et al.* 1999b). *Phytophthora cinnamomi* populations from France, New Zealand and Western Australia also exhibited variation in virulence on forestry tree species (CHEE and NEWHOOK 1964; HÜBERLI *et al.* 2001; ROBIN and DESPREZ-LOUSTAU 1998). A collection of 12 *P. cinnamomi* isolates from the Limpopo, Mpumalanga and KwaZulu-Natal provinces in South Africa and California in the USA were evaluated for sporulation, growth rate and virulence on avocado stems.

Differences in growth rate on 5% V8 agar, half strength potato dextrose agar (PDA) and malt extract agar (MEA), sporulation and virulence to, and colonization of avocado were observed between different *P. cinnamomi* isolates, with no single isolate outperforming others in all experiments. Some isolates, such as HB2T12R1 grew and sporulated well, but displayed average virulence on avocado, while others, such as FVB6T2S4 and GKB4T9S4 exhibited above average sporulation abilities and virulence, but slow growth rates. Geographical origin of isolates had no effect on phenotypic variation, as was the case in the most recent published report of phenotypical variation among South African *P. cinnamomi* isolates (LINDE *et al.* 1999b).

This dissertation contributed to the identification of five *P. cinnamomi* isolates, FVB6T2S4, GKB4T9S4, HB2T12R1, HB5T11R2 and 942 from Mpumalanga, Limpopo and the USA for use in infection studies and screening of resistant avocado rootstocks. This subset represents isolates that grow well in culture, and have a high sporulation ability and virulence to avocado. Isolate 942 was present at significantly higher levels in lesions as seen from pathogen quantification results, and was included in the subset of isolates for infection trials due to its superior colonization ability. Promising new avocado rootstock selections should be challenged with a mixed inoculum to ensure they are tolerant or resistant to the whole pathogen population to ensure durability of resistance (LINDE *et al.* 1999b; ROBIN and DESPREZ-LOUSTAU 1998). More *P. cinnamomi* isolates from avocado orchards should be evaluated for differences in traits such as virulence, growth rate and sporulation, and the addition of mating type and genotypic data will contribute towards the use of well-characterized isolates in infection, microscopy and gene expression studies, as well as transformation and rootstock screening trials.

The findings of this study contributed towards the establishment of a protoplast transformation procedure for *P. cinnamomi*. Optimization of protoplast production and regeneration of *P. cinnamomi* reported in this study will serve as a platform for further attempts at establishing a protoplast transformation protocol for *P. cinnamomi.* Possible isolate-specific effects of transformation were highlighted, and should be considered in future transformation attempts. Confirmation of variation in pathogenicity among *P. cinnamomi* isolates from avocado orchards is of value to both the Fruit Tree Biotechnology Programme and Westfalia Technological Services. The phenotypic variation observed illustrates the importance of using mixed inoculums in infection and screening trials.

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Summary

Phytophthora root rot (PRR) caused by *Phytophthora cinnamomi* is the major limiting factor of avocado production in South Africa. Good orchard management practices such as mulching and chemical control through the use of phosphite contributes to disease control, but the use of rootstocks tolerant to PRR are the most effective. Investigation into the interaction between avocado and *P. cinnamomi* on both a physiological and molecular level is required to identify the plant mechanisms responsible for tolerance to PRR in some rootstocks. The firm aim of this study was to establish a transformation system for *P. cinnamomi*, to allow for the production of fluorescently tagged strains of *P. cinnamomi* in order to study the interaction between *P. cinnamomi* and avocado *in planta*.

A number of protocols used for transformation of *P. cinnamomi* and other species such as *P. infestans* and *P. sojae* were tested, and two putative GFP transformants were obtained. Neither transformant emitted fluorescence, and this could have been due to loss or silencing of the transgene. The establishment of a transformation protocol is not only important for studying the host-pathogen interaction, but is also required for functional validation of approximately 2000 putative *P. cinnamomi* pathogenicity genes recently identified. *Agrobacterium tumefaciens*-mediated transformation or a biolistics approach could be considered in future if the efficiency of protoplast mediated transformation cannot be improved.

This was followed by re-evaluation of the amount of phenotypic variation in South African *P. cinnamomi* isolates, with a focus on isolates obtained from avocado orchards in the Limpopo, Mpumalanga and KwaZulu-Natal provinces in South Africa and California, USA. Sporulation ability, *in vitro* growth rate and virulence on avocado were assessed, and significant differences were observed between different isolates. No single isolate performed the best across all experiments, and geographic origin of isolates did not influence phenotypic characteristics. These results were consistent with results of previous studies, which found variation in traits associated with pathogenicity in clonal South African *P. cinnamomi* populations.

The results led to the recommendation that a mixed inoculum, consisting of isolates representing good sporulators and growers, as well as isolates that are highly virulent to avocado, should be used in infection and rootstock screening trials. It is widely accepted that the durability of resistance in crops is dependent on the amount of variation present in pathogen populations. This subset of isolates serve as a representative of the phenotypic

variation possible present in entire populations, and will ensure that results obtained in trials will be applicable in a larger context.