

**Determining the phenotypic resistance
mechanisms in avocado against
*Phytophthora cinnamomi***

Barry Christie

**Determining the phenotypic resistance
mechanisms in avocado against
*Phytophthora cinnamomi***

by

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Declaration

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

A handwritten signature in black ink, appearing to be 'Barry Christie', written over a horizontal line.

Barry Christie

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Preface

The avocado (*Persea americana* Mill.) is an economically important crop worldwide. The most important disease of avocado is Phytophthora root rot, which is caused by *Phytophthora cinnamomi* Rands. Currently, phosphonate trunk injections provide satisfactory disease control; however, the possibility of reduced sensitivity and eventually resistance to this fungicide is lurking on the horizon. Furthermore, consumer demands for “organic” fruit has been increasing over the past decade, emphasising the need to use root rot-resistant rootstocks. Due to a lack of understanding of the interaction between these two organisms, screening for specific resistant mechanisms is not possible and consequently only partially resistant rootstocks are currently commercially available. The aim of this thesis was therefore to address this need by investigating phenotypic traits in avocado rootstocks that could play a role in resistance against *P. cinnamomi*.

Chapter 1, entitled “*Phytophthora cinnamomi* and the avocado: Infection strategies and defense mechanisms”, provides a comprehensive review of past and recent literature. A brief overview is given of the avocado, followed by basic biology and infection strategies of *P. cinnamomi*. The focus of this chapter, however, is on resistance mechanisms that plants use against *Phytophthora* spp., and light is in particular shed on histological defense followed by a biochemical defense section.

Throughout this dissertation, three avocado rootstocks which vary in resistance to *P. cinnamomi* are compared in the various experiments, except where otherwise stated. In **Chapter 2**, attention is given to visible phenotypic trait differences by means of microscopy. Morphological and anatomical comparisons were made between rootstocks using stereo- and light microscopy, respectively. The infection process of the pathogen was described externally, as was the ability of the various rootstocks to suppress zoospore attraction and cyst germination with scanning electron microscopy. The second chapter also describes

infection internally, including observations of how the three rootstocks responded upon infection, using confocal laser scanning microscopy.

Chapter 3 describes how the three avocado rootstocks respond biochemically to *P. cinnamomi* infection. Total phenolic content comparisons were made between rootstocks that were planted in a field trial, as well as in a mistbed-inoculation trial. Here was also compared the response of reactive oxygen species-scavenging enzymes, namely ascorbate- and guaiacol peroxidase, catalase and superoxide dismutase. Finally, the change in activity of β -1,3-glucanase, an enzyme that is known to inhibit *P. cinnamomi* cyst germination, is also described.

A general discussion is provided in **Chapter 4**, where results from the two experimental chapters are discussed and correlated. Ideas and future opportunities to improve understandings of this complex host-pathogen interaction are shared in this last chapter.

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List of Abbreviations

% - Percent

°C – Degrees Celsius

μl – Microliter

μm – Micrometer

ANOVA – Analysis of variance

APX – Ascorbate peroxidase

AsA – Ascorbate

CaCO₃ – Calcium carbonate

CLSM – Confocal laser scanning microscopy

cm – Centimeter

Cu – Copper

CWBPA – Cell wall bound phenolic acids

dH₂O – Distilled water

dpi – Days post inoculation

E – Extinction coefficient

EBPA – Ester bound phenolic acids

ELISA – Enzyme-linked immunosorbent assay

FAA – Formalin acetic acid alcohol

g – Gram

g – gram

GBPA – Glycoside bound phenolic acids

GIPs – Glucanase inhibiting proteins

GPX – Guaiacol peroxidase

h – hour(s)

H₂O – Water

H₂O₂ – Hydrogen peroxide

HCl – Hydrochloric acid

hpi – Hours post inoculation

HR – Hypersensitive response

IBA - Indolebutyric acid
KCl – Potassium chloride
kg – kilogram
kPa – Kilo Pascal
L – Liter
M – Molar
mg – milligram
min – minute
ml – milliliter
mm – Millimeter
mM – Millimolar
Mr. – Mister
Na₂CO₃ – Sodium carbonate
NaCl – Sodium chloride
NCPA – Non-conjugated phenolic acids
NH₄Cl – Ammonium chloride
nm – nanometer
PAMPS – Pathogen-associated molecular patterns
PARPH – pimaricin; ampicillin; rifampicin; pentachloronitrobenzene
PCD – Programmed cell death
PCR – Polymerase chain reaction
PDA – Potato dextrose agar
PRR – Phytophthora root rot
ROS – Reactive oxygen species
rpm – Revolutions per minute
SEM – Scanning electron microscopy
SOD – Superoxide dismutase
spp. – Species
TMV – Tobacco mosaic virus
TSPA – Total soluble phenolic acids
U – Units

UV – Ultraviolet

v – Volume

w – Weight

XTT – 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

Zn – Zinc

Chapter 1

***Phytophthora cinnamomi* and the avocado: Infection strategies and defense mechanisms**

Introduction

For the past 9000 years, avocado, *Persea americana* Mill., has been consumed in Mesoamerica. Archaeological evidence suggests that selection for larger fruit began as early as 900 B. C. in Mesoamerica, its region of likely origin (Smith, 1966; Whiley *et al.*, 2002). With a global production in 2005 of 3 413 984 tonnes, valued at 1 098 742 million USD, and a global production of 3 555 265 tonnes in 2008 (FAOSTAT, 2010), the avocado is an economically important crop.

The pathogen *Phytophthora cinnamomi* Rands is a major threat to the avocado industry worldwide, causing root rot and cankers. Since the discovery of the *Phytophthora* genus (De Bary, 1876), attempts have been made to understand the biology, epidemiology and behaviour of these pathogens and the control of the diseases that they cause. Although the avocado plant is severely affected by root rot, very specific defense mechanisms against *P. cinnamomi* are not likely (Whiley *et al.*, 2002). However, the fact remains that certain avocado cultivars are more tolerant to *Phytophthora* root rot (PRR). Although the mechanisms of resistance or tolerance are not completely understood, their enhanced understanding would facilitate control of the disease.

Plants and pathogens compete with each other by developing specific attack or defense strategies in order to survive. For this reason non-host reactions are much more common and disease is the exception (Maor & Shirasu, 2005; Stahl & Bishop, 2000). Models for understanding host-pathogen interactions have been developed from the interactions of *Phytophthora* species with plants, since species within this genus represent some of the most ecologically and economically important plant pathogens (Hammerschmidt, 2008). Using these models, as well as incorporating studies which specifically focused on the interaction of *Phytophthora* spp. and host plants, including *P. cinnamomi* and avocado, this review will report on mechanisms for pathogen attack and plant defense.

The Host: Avocado

Background

The avocado (family Lauraceae), is a polymorphic species with three recognized, cross-fertile subspecies, namely *P. americana* var. *americana* (West Indian), *P. americana* var. *guatemalensis* L. Wms. (Guatemalan) and *P. americana* var. *drymifolia* Blake (Mexican; Whiley *et al.*, 2002). The avocado only became a commercial crop after 'Fuerte' was selected from Mexico and introduced to California in 1911. Avocados are grown commercially in almost 50 countries (Demirkol, 1995) and are important fruit to countries and states such as California, Hawaii, Florida, Puerto Rico, Mexico, Dominican Republic, Chile, Brazil, Indonesia, South Africa, Venezuela, Australia, Spain, France, Guam, Samoa, and Israel (Demirkol, 1995; Whiley *et al.*, 2002). In the late 19th century the first avocado trees were brought to South Africa (KwaZulu Natal), probably of the West Indian race (Ben-Ya'acov & Michelson, 1995; Whiley *et al.*, 2002).

Avocado rootstocks

Although South Africa is a major avocado exporter, research has not been done on avocado rootstocks to the desired extent. Avocado rootstocks play a crucial role in tree vigour, production and even resistance to certain fruit diseases (Ben-Ya'acov *et al.*, 1993; Willingham *et al.*, 2001; Wolstenholme, 2003). Westfalia Technological Services started a rootstock breeding programme in the 1980s, led by Dr Sylvie Kremer-Köhne, and has since contributed significantly to research and development of PRR-resistant rootstocks in South Africa, of which the widely-known 'Dusa[®]' rootstock is an example (Kremer-Köhne & Köhne, 1992; Kremer-Köhne & Duvenhage, 2000; Köhne, 2005).

The first reported avocado rootstock selection programme was established in California by F. F. Halma in the early 1940s, but was focused on productivity, rather than PRR resistance. Only later, when *P. cinnamomi* was classified as a vigorous pathogen of the avocado, did G.

A. Zentmyer begin selection of rootstocks for PRR resistance (Ben-Ya'acov & Michelson, 1995; Whiley *et al.*, 2002). Research on clonal avocado rootstocks has become a major focus in California and other countries, especially in the past few decades. In California, it was not until the end of the 1950s, that scions were commercially being grafted onto seedling rootstocks (Ben-Ya'acov & Michelson, 1995). The advantages and disadvantages of seedling and clonal rootstocks will be discussed.

Seedling rootstocks

Seedling rootstocks are derived from the germination of seeds (often 'Edranol'), whereafter the scion cultivar is grafted onto the seedling. Seedling rootstocks are hybrids between the mother and the pollen parent and, thus, result in non-uniformity of orchards. However, seedling rootstocks usually establish more quickly in the field than clonal rootstocks (Whiley *et al.*, 1990). Other advantages of seedling propagation include low costs, less time and ease of propagation. Seedling rootstocks allow for a high genetic diversity in an orchard.

Seedling rootstocks are still used in much of the world. In Australia, Ben-Ya'acov and Michelson (1995) reported that only approximately 2% of the rootstocks were clonal. In early years South Africa only planted seedlings of Mexican origin but later used 'Edranol' (Guatemalan) seedlings as rootstocks. In Mexico, seedling rootstocks are common and clonal rootstock production only commenced recently (Ben-Ya'acov & Michelson, 1995).

Clonal rootstocks

Clonal rootstocks are, as the name implies, derived from a desired clone of a selected avocado "cultivar" and used as a rooting system, rather than for fruiting characteristics. Less than 1% of the seed of a PRR resistant tree inherit the resistance of the parent tree, rendering clonal propagation the only means of producing PRR-resistant material for commercial use. Coffey and Guillemet (1987) pointed out that resistance to PRR is the main

economical factor determining the choice of avocado rootstock. However, grafting compatibility, tree vigour and hence the production capacities are also important when considering the choice of rootstock (personal communication, JS Köhne; Ben-Ya'acov & Michelson, 1995; Köhne, 2005). A desired rootstock is usually saline and PRR tolerant and highly productive. Clonal rootstocks allow uniformity in an orchard, making management practices more simple and economical.

Various risks are imposed when planting clonal material. Pathogen mutations, hybridization and sexual reproduction may all lead to genetic recombination, which in turn allow pathogens to overcome a tree's resistance (Agrios, 2005; Brasier *et al.*, 1999). When a single clone is susceptible to a specific isolate, race or species of pathogen or pest, large economic damages could occur. The PRR tolerant rootstock 'Duke 6' is such an example, and was completely abandoned due to it being susceptible to stem pitting in South Africa in the late 1980s (Moll *et al.*, 1987). Risks are not only linked to biological threats, but also abiotic stresses such as drought, flooding, chilling, fires, etc (Agrios, 2005).

In South Africa clonal avocado rootstock propagation was first conducted in the late 1970s at Westfalia Estate (Köhne, 2005). Various clonal propagation techniques are available. A widespread technique of clonal rootstock propagation used in South Africa is a modification of the "Brokaw" and the "Frolich" technique, developed by Ernst (1999). This is carried out by grafting the desired rootstock onto a seedling plantlet derived from a nurse seed (usually 'Zutano' or 'Edranol').

Modern grafting steps involve a combination of various techniques. The seedling is grown in a polyethylene bag (350 x 100 mm; 1 liter content) containing sterile composted pine bark. Once the stem is thick enough (approximately 6 mm) it is grafted with the desired rootstock, followed by an etiolation step (dark room with good ventilation at approximately 25°C) at bud burst. Up to two shoots per graft are allowed to develop. Once shoots have reached a length of 200 to 300 mm the plants are removed from the etiolation room. Indolebutyric acid (IBA; other hormones are also used) is applied to a small incision approximately 100

mm above the graft union, and 2 g Plantocote 8M (slow release fertilizer) can be applied on top of the growth medium. A washer or wire is fit around the stem above the seed to sever the vascular cambium to accumulate hormones for root initiation and starve the nurse seed. The bags are filled with rooting medium and placed under shade for root and shoot growth. When sufficient shoot growth has occurred, the desired scion cultivar can be grafted onto the clonal cultivar (Ernst, 1999; Whiley *et al.*, 2002; personal communication, JS Köhne).

Several clonal rootstocks (Table 1.1) are available commercially, such as 'Thomas', 'Toro Canyon', 'Jovo', 'Spencer' and 'Duke 7', the latter of which is the first commercial standard rootstock with moderate resistance against PRR. In 2001 a new clonal rootstock, 'Merensky 2' or 'Dusa[®]', was released for commercial use by Merensky Technological Services (currently known as Westfalia Technological Services). 'Dusa[®]' is in all probability of Guatemalan and Mexican origin (Köhne, 2005; Whiley *et al.*, 2002) and outperforms 'Duke 7' in many areas with regard to tree vigour, 'Hass' yield, tolerance to cold, resistance to PRR and performance in saline soils (Wolstenholme, 2003).

Avocado root anatomy and development

Dicotylodenous root anatomy is defined by a few major constituents, namely the endodermis surrounding the stele in the center of the root, which is in turn surrounded by a cortex area, and lastly an epidermis, a thin layer of cells towards the outside of the root. The stele consists primarily of xylem, phloem and pericycle and is surrounded by the endodermis, which is not always very evident in avocado roots. The endodermis, primarily consisting of parenchyma cells, is in turn surrounded by the epidermis (Figure 1.1; Fassio *et al.*, 2007).

Early literature reports the absence of root hairs on avocado roots (Ginsburg & Avizohar-Hershenson, 1965). To the author's knowledge, the first anatomical study on avocado roots was conducted by Heismann (1939). This initial study reported that avocado roots are

tetrarch, implicating that there are four xylem strands occurring in the stele of the root (Figure 1.2). However, avocado roots vary in xylem anatomy structures, even within the same cultivars. As root diameter increases with more secondary growth, the stele becomes proportionally more important, with less cortex area. As secondary growth continues, the vascular tissue matures and the formation of a periderm commences, which are visible in the external layers of the pericycle. As growth continues, the cortex becomes displaced and in proportion to total root area becomes negligible. Lignification also commences, and the thickening of the epidermis also becomes apparent (Fassio *et al.*, 2007).

The pathogen: *Phytophthora cinnamomi*

Background

The Genus *Phytophthora* was first described by Professor Anton de Bary, from the University of Strasbourg in 1876, and gained prominence only after the discovery that the Irish potato famine was caused by the species *Phytophthora infestans* de Bary (De Bary, 1876; Ribeiro *et al.*, 1978). *Phytophthora* belongs to the Stramenopiles kingdom (formerly Chromista), phylum Oomycota, class Oomycetes, order Peronosporales and the Pythiaceae family.

Origin

Although there is much speculation on its centre of origin, *P. cinnamomi* is believed to have originated from Southeast Asia (Papua New Guinea, Indonesia, Taiwan and Malaysia) and probably spread from there to other parts of the world through human activities (Hardham, 2005; Linde *et al.*, 1999; Pratt & Heather, 1973; Whiley *et al.*, 2002; Zentmyer, 1985). The annual mean temperatures for Papua New Guinea range from 21.1 to 32.2°C and annual rainfall of above 5000 mm occurs (Microsoft® Encarta® 2006) which is perhaps the ideal environmental conditions for the development of the pathogen. However, *P. cinnamomi*

was first described and isolated in 1922 from cinnamon trees (*Cinnamomum burmanii* Nees; hence its name) in the mountains of West Sumatra by R.D. Rands (Rands, 1922).

Diversity

Two mating types are found in *Phytophthora* species, namely A1 and A2. Although both mating types are often found in the same regions, sexual reproduction occurs very seldom. Three clonal lineages of *P. cinnamomi* occur in South Africa, Australia and other regions around the world (Dobrowolski *et al.*, 2003), substantiating the fact that little genetic recombination occurs (Hardham, 2005). Linde *et al.* (1999) compared the gene and genotypic diversity of Australian and South African isolates of *P. cinnamomi* and found very little variation. However, despite the fact that virtually no crossing between mating types occur in the field, *P. cinnamomi* is capable of adapting to new environments and hosts by means of mitotic recombination (Hardham, 2005).

Biology

Rands (1922) studied symptoms of stripe canker on the cinnamon trees from which the pathogen was isolated, culture behaviour of the pathogen as well as its morphology. *Phytophthora cinnamomi* is capable of growing on a diversity of culture media, and has even been observed to produce sporangia on cooked flies. This initial comprehensive report also described the different reproduction structures that are produced by this pathogen, such as chlamydospores, zoosporangia and zoospores. Chlamydospores are spherical in shape and have walls of 2-2.5 µm in thickness. Chlamydospores are asexual structures and form readily in many artificial media, as well as naturally in infected plant tissue. Zoosporangia can form on oospores, somatic hyphae or even from chlamydospores (Hardham, 2005).

Rands (1922) observed the morphology and structure of zoospores and described two flagella present on the zoospores, which enable them to swim to infection sites (Zentmyer,

1955). Hardham (1987) documented the ultrastructure of *P. cinnamomi* zoospores and noted that they are ovoid and biflagellate, but lack cell walls. A high hydrostatic pressure within zoosporangia results in zoospore release (Gisi *et al.*, 1980). The host is located via chemotaxis and encystment of zoospores occurs within minutes (Hardham, 1985; Hardham, 1987), whereafter infection follows.

Host / *Phytophthora* spp. interactions

Host diversity

Hosts on which *P. cinnamomi* occurs include: *Ananas comosus* L., *Castanea dentata* L., *Castanea sativa* Mill., *Cinchona* spp., *Chamaecyparis lawsoniana* A. Murr., *Cinnamomum* spp., conifers, *Ericaceae*, *Eucalyptus* spp., *Fagus* spp., *Juglans* spp., *Pinus* spp., *Prunus* spp., *Quercus* spp., avocado and many more species (OEPP/EPPO, 2004). In 1955 a total of 116 species were reported as hosts of *P. cinnamomi* (Zentmyer, 1955), but has since been found on more than 3000 plant species (Hardham, 2005). *Phytophthora cinnamomi* preferentially attacks the feeder roots of many of the hosts it infects. However, *P. cinnamomi* causes major cankers on trunks, crowns and major roots of crops such as macadamia (*Macadamia integrifolia* Maiden & Betche). Zentmyer (1951) stated that all but one of the diseases of *P. americana* are caused by fungi (although *P. cinnamomi* has since been classified as an oomycete), of which PRR is the most important in most areas (Zentmyer *et al.*, 1951).

The role of chemo-attraction and root exudates

The earliest indication that the symptoms of PRR of avocado were caused by an agent other than excess water in the soil was in 1929 when Tucker continually isolated *P. cinnamomi* from infected roots in Puerto Rico (Tucker, 1929; Zentmyer, 1955). In South Africa, Wager (1931) studied the disease on avocado, but the causal agent was identified as *P. cambivora* (Petri) Buisman. Although Tucker (1929) had already described the causal agent of PRR of

avocado as *P. cinnamomi*, Wager (1931) stated that a certain Mr. Ashby identified the *P. cinnamomi* isolates of Tucker (1929) as *P. cambivora*, which was a misidentification. In the United States, PRR was reported for the first time by Wager (1942). However, in 1944, Parker and Rounds compiled a report on the relationship between soil moisture content, its drainage, and avocado tree decline. Although publications on PRR were referenced in the article, the pathogen was not mentioned once in the report (Parker & Rounds, 1944). Zentmyer (1955) also concluded that excess soil moisture is a necessity for this oomycete in order to infect trees. These series of observations and reports lead to an understanding that soil moisture content and chemotaxis might play a role in the PRR disease triangle.

Since infection depends on excess water (a soil matric potential of 10 kPa or less), and is not a result of chance or the distribution of zoospores in the soil, a tropism towards a target needs to be implemented by the pathogen in order to reach the roots (personal communication, K.G. Pegg). In fact this has been proven to be true for the chemotactic responses of *P. cinnamomi* on avocado (Botha & Kotze, 1989; Zentmyer, 1961a; Zentmyer, 1961b) as well as *Phytophthora* spp. on other hosts (Morris & Ward, 1992). Germ tubes of germinating zoospores were also observed to have a positive tropism towards avocado roots (Zentmyer, 1961b). Reduced root exudates to attract zoospores are thought to be a mechanism to limit infection of roots in PRR resistant rootstocks (personal communication, K.G. Pegg).

Oomycete–plant interactions depend on the specific combination of host and pathogen species. In a comparative study by Raftoyannis and Dick (2006) between several host species and oomycetes (*Pythium* and *Phytophthora* spp.), very little correlation could be found between disease severity and the amount of encysted zoospores. The results of their study indicated that of the tested species *Pythium coloratum* Vaartaja, *Pythium deliense* Meurs, *Pythium diclinum* Tokunaga and *Phytophthora nicotianae* van Breda de Haan were the only pathogens that showed a positive correlation between disease incidence and amount of cysts in various hosts (Raftoyannis & Dick, 2006). *Phytophthora cinnamomi* zoospores were

shown to have tropism towards tips and cut ends of citrus, macadamia and pea roots, but not to the area of elongation as in the case of avocado roots. Chemotaxis of a citrus root pathogen, *Phytophthora citrophthora* Sm. & Sm., was also investigated on avocado roots, and no attraction was observed, however chemo-attraction was evident on citrus roots (Zentmyer, 1961b).

Chemotaxis of zoospores is a well-known phenomenon where zoospores are attracted towards a specific attractant. Interestingly, Allen and Harvey (1974) studied the behaviour of zoospores, but proved that negative chemotaxis can also occur. A series of concentration gradients were used for the compounds HCl, KCl, NaCl and NH₄Cl and the critical concentration of each cation was determined (Allen & Harvey, 1974). The zoospores of the economically important pathogen *Phytophthora sojae* Kaufmann & Gerdemann are attracted to isoflavones such as daidzein and genistein which are excreted by soybean roots (Morris & Ward, 1992). Morris *et al.* (1998) suggested that specific isoflavones (daidzein and genistein) aids in the chemo-tropism of *P. sojae* mycelia to soybean roots (Morris *et al.*, 1998). The root exudates of avocados have been shown to play a role in the chemo-attraction of *P. cinnamomi* zoospores (Zentmyer, 1961a). Interestingly, the work of Zentmyer (1961b) showed that the chemo-attraction of zoospores towards avocado roots is only prevalent when the roots are alive. This was shown by boiling roots before inoculation with zoospores. Botha and Kotze (1989) demonstrated that zoospores are attracted in higher quantities to roots of susceptible avocado rootstocks (such as 'Edranol' seedlings) than to more resistant rootstocks such as 'Duke 7', 'G6' and 'G755'. Amino acids excreted from avocado roots were analysed in the study and found to be excreted in a significantly higher amount in the susceptible 'Edranol' cultivar than the other three resistant rootstocks. The most remarkable attraction of zoospores occurred when a combination of all 14 tested amino acids were used, whereas individual amino acids did not influence the chemo-attraction of zoospores significantly, with the exception of glutamic acid. It was also shown that the *P. cinnamomi* resistant rootstock 'G755', although not very productive, attracted significantly fewer zoospores than 'Edranol' and 'Duke 7' (Botha & Kotze, 1989).

Histopathology of roots

Histopathology in plants is the study of a pathogen's interaction with the host plant at the cellular level. Upon infection, plants usually respond in a very specific way, depending on the host and the pathogen. These responses are then measured. For instance, Braun and Sinclair (1976) used light and electron microscopy to examine histological changes that occurred in American elm, *Ulmus americana* L., affected by phloem necrosis. Often, there are differences between resistant and susceptible cultivars (Beagle-Ristaino & Rissler, 1983; Braun & Sinclair, 1976; Rahman *et al.*, 1999).

After host location via chemotaxis, zoospores of *Phytophthora* spp. attach and encyst. In a study by Oh and Hansen (2007), it was observed that the zoospores of *Phytophthora lateralis* Tucker & Milbrath germinated at similar frequencies on resistant and susceptible roots of Port-Orford-cedar (POC, *Chamaecyparis lawsoniana* A. Murray Parl.), but the penetration frequency was lower on the roots of resistant plants. Cell wall thickening was observed in resistant roots, whereas no thickening occurred in susceptible roots. These thickenings even occurred in cells distant from the observed hyphae (Oh & Hansen, 2007). In the same study it was also found that cell collapse, consistent with a hypersensitive response (HR), coincided with infection. It is, however, not known whether this cell collapse is a result of cellular degradation by the pathogen, or whether it is truly part of the HR reaction and programmed cell death.

For many years it was thought that *P. cinnamomi* could not penetrate suberized tissue. O' Gara *et al.* (1996) reported wilting in 1-year-old jarrah trees (*Eucalyptus marginata* Sm.) 6 days after inoculation, regardless of whether stems were wounded or not. Thus, infection through unwounded stems appears to be possible, and may be why *Phytophthora* cankers develop on avocado trees and other crops. Whether older, non-wounded jarrah trees could be infected is not known (O'Gara *et al.*, 1996).

Phytophthora cinnamomi aggressively penetrates the roots of the many hosts it infects. In a comparative study by Legge *et al.* (1989) it was demonstrated that *P. cinnamomi* zoospores germinated, encysted and penetrated roots of both susceptible and resistant plants. In the same study lesions that indicated infection could be detected within 8-16 h after invasion on all species and root growth ceased after 24 h, but growth resumed from lateral roots within 48 h in resistant species. Cellular responses to infection in different species varying in susceptibility differed considerably. Species resistant to *P. cinnamomi* (*Eucalyptus calophylla* R. Br. Ex Lindl, *Eucalyptus maculata* Hook., *Acacia pulchella* R. Br., *Gahnia radula* (R. Br.) Benth., *Juncus bufonius* L., *Zea mays* L., *Triticum aestivum* L.) did not kill the pathogen. In spite of the fact that lesion development was restricted and root growth resumed, the pathogen could still be isolated. Histological responses were restricted to the areas with lesions; for instance, *E. calophylla* produced new roots just above the lesion that did not develop symptoms. Phenolic or tannin-like substances were also observed, indicating that biochemical compounds play a role in the defense response. Individual cells of *Acacia pulchella* died and consequently created a barrier between healthy and dead tissue (Legge *et al.*, 1989).

Tippett & Malajczuk (1979) were the first to report a completely resistant host (*A. pulchella* response to *P. cinnamomi*). Interestingly, zoospores were still attracted to the roots at the zone of elongation, and rapid germination was observed. Germ tubes penetrated the cortex as far as three cell layers. Total disruption of invaded cells, or cells adjacent to hyphae or germ tubes, took place 4-7 h after infection. Rapid death of these cells occurred and was associated with a dark stained material, presumably phenolic compounds. Due to the sharp demarcation between healthy and dead cells, an HR was suggested. In the root tip at the area of cell differentiation, penetrated germ tubes were mainly observed to grow intercellularly; however, intracellular germ tubes or hyphae were also observed, but only in dead cortical cells. Other observations in the study included electron-dense droplet-like substances that lined the vacuolar membranes and a “tanned” cytoplasm in cells that were undergoing rapid necrosis. Cell membranes were reported to occasionally be separated from the cell walls. Necrotic cells also showed wall appositions next to intercellular hyphae.

These cell wall appositions were suggested to be callose deposits. 24-26 h after inoculation with a high zoospore concentration, swollen and a tubular endoplasmic reticulum was observed in cells next to cells undergoing necrosis. Tippet & Malajczuk (1979) suggested that this indicated increased callose production, as well as increased secretory activity. A similar response was observed in maize (*Z. mays*) where a distinct difference in response was found after being challenged with *P. cinnamomi*, when compared to *Lupinus angustifolius* L., which is susceptible and often used as *Phytophthora* bait. Maize roots produced callose 2-4 h after infection, whereas no callose formation was present in *L. angustifolius* (Hinch & Clarke, 1982). Noteworthy of the study by Hinch and Clarke (1982) is the fact that not all cells produced callose, and that extracellular components were likely to be responsible for the induction of callose formation.

In an attempt to simulate natural infection by keeping the roots attached to the plants, Aveling and Rijkenberg (1986) inoculated a susceptible Mexican avocado seedling. Roots were dipped into a zoospore suspension and harvested after 0.5, 1, 4, 8, 24 and 48 h. Zoospores were mainly attracted to the cell elongation area just behind the root tip, as well as to wounded sites. Zoospores were observed to encyst, germinate and formed appressoria-like structures before penetrating the roots, but direct penetration also occurred. Intercellular penetration through the epidermis was observed most frequently and hyphae successfully entered the cortex in the area of root elongation. Hyphae were observed to be constricted at points close to the penetrated cell wall, but inside cells they were normal size and hyphae almost filled the cellular lumen after branching occurred. Rapid disruption of cells adjacent to intercellular hyphae was also visible. The study revealed that a visible host response occurred 8 h after infection with phenolic inclusions accumulating a short distance in advance of infected areas (Aveling & Rijkenberg, 1986). Zoospore encystment and germination were investigated by scanning electron microscopy as well as light microscopy. Aveling and Rijkenberg (1991) found similar results to their previous study (Aveling & Rijkenberg, 1986), where penetration of roots occurred more frequently via germ tube apices than by the formation of appressoria-like structures. The study revealed that not only were fewer zoospores encysted on the tolerant rootstock 'Martin Grande', but that fewer zoospores germinated on tolerant avocado roots than on

those of the susceptible 'Edranol'. This suggests either a lack of host recognition or some form of biochemical defense that is exhibited by the plant roots (Aveling & Rijkenberg, 1991). Aveling and Rijkenberg (1989) concluded their study by suggesting that post-penetration mechanisms in the cultivars 'Duke 7', 'G6' and 'Martin Grande' are likely to be involved in the tolerance of these rootstocks.

An infection study on avocados was done by examining the cellular responses of 'Duke 7' after being challenged by *P. cinnamomi* (Phillips et al., 1987). Zoospore-inoculated root tips developed lesions within 24-48 h. Initial observations showed that lesion formation ceased in tissues that were lignified and had undergone secondary thickening. Although lesion development stopped, *P. cinnamomi* could always be re-isolated 6 mm ahead of the lesion edge.

Healthy, uninoculated control avocado roots that were examined microscopically in the study by Phillips *et al.* (1987) showed well-differentiated vascular development by secondary wall thickening and lignification. Cells in the region of elongation and the root cap contained large vacuoles, which were thought to have a secretory function; the area of cell elongation is the major region to which zoospores are attracted, due to organic compounds that leak from this area. Away from the provascular tissue, cells became progressively more vacuolated. The uninoculated roots also showed a concentration of starch grains near the root tips and tyloses were observed in xylem bundles, although infrequently. Compared to infected roots, control roots did not show the presence of phenols and tannins in the area between the root tip and differentiated tissue. Tylose formation was infrequent. Inoculated roots were characterized by three distinct zones, although the edges were not sharply defined. Tissue necrosis was prevalent in the first zone, which extended from the point of inoculation just behind the root tip into the area where cell differentiation had taken place. *Phytophthora cinnamomi* hyphae grew inter- and intracellularly through the cortex and stellar tissue. Cells in the cortex and pericycle were scattered and cell walls were degraded. Tannins and phenolic compounds were abundant in the stellar and cortical tissue. A reduction in lignification in the xylem vessels and xylem parenchyma were observed and mainly constricted to larger metaxylem vessels. Tyloses were present along the length of the

xylem vessels, completely blocked the vessel lumen, and also arose from the axial parenchyma cells. The second zone was characterized by changes in both hyphal growth as well as root anatomy. A distinctly smaller region in the cortex was affected and hyphal growth and development was also limited. Thickened cell walls and intercellular spaces (thickened with an amorphous material) were observed in the layer of cells directly adjacent to infected tissue. This layer was defined as the necrophylactic periderm, and extended from the epidermis to the endodermis. Stimulated periclinal cell wall divisions in the phloem bundles resulted in whorls of cells, which walled off infected bundles surrounding the lesions. The third zone extended upwards from 1-2 cm ahead of the lesion end point. Although most cellular responses as described above were absent in the third zone, tyloses were still present for up to 3.5 cm ahead of the lesion end point. The study revealed that the lesion formation was limited in inoculated roots; however, the mycelia of *P. cinnamomi* were detected at the distal end of the altered structures, indicating that biochemical defense plays a role in the resistance mechanisms of 'Duke 7' (Phillips *et al.*, 1987).

Tolerance and vigour

Apart from the responses of plants to infection, tree vigour could also influence the welfare of the host. The anatomy of the xylem vessels in roots influence the uptake of water and consequently also mineral nutrients (Fassio *et al.* 2009). Different avocado rootstocks differ in anatomy. Fassio *et al.* (2009) showed that 'Duke 7' had a significant lower amount of xylem vessels than 'Toro Canyon', a higher average xylem vessel area and had 19% more total conduit area than 'Toro Canyon'. Whether this phenomenon influences root rot resistance in avocados has not been determined. Some rootstocks tolerate PRR via the constant production of new, healthy feeder roots once older roots are infected (Ben-Ya'acov & Michelson, 1995).

Biochemical defense responses against *Phytophthora* spp.

Biochemical defense is a common phenomenon in many host-pathogen interactions, and many crops depend on these mechanisms in order to overcome the devastating effects of plant pathogens. These mechanisms for defense include the production and secretion of various molecules in plants such as phenolic compounds (Baayen *et al.*, 1989; De Ascensao & Dubery, 2003; Phillips *et al.*, 1987), the initiation of the HR that precedes many defense-related proteins (Oh & Hansen, 2007; Tippet & Malajczuk, 1979), enzymes such as cellulases and peroxidases (Hammerschmidt *et al.*, 1982), and the production of superoxide dismutase, reactive oxygen species (ROS), catalase, phenylalanine ammonia lyase and polyphenol oxidase (Subramaniam *et al.*, 2006).

Phenolic compounds include a wide range of molecules which possess an aromatic ring, containing a hydroxyl substituent and usually a carbon chain containing a carboxyl group (R-COOH; Thomson, 1964) as well as a methoxyl (-O-CH₃) group. What should be noted is that the presence of phenolic acids in plants does not necessarily contribute to resistance in plants, and that phenolics in low concentrations could even stimulate pathogen growth (Cruickshank & Perrin, 1964). Phenolic compounds are present in plants in different forms and are part of the defense response in the host-pathogen interaction in many plants. In strawberry fruit, phenolics were shown to inhibit *Botrytis cinerea* Pers. and *Cladosporium cladosporioides* (Fres.) de Vries. (Terry *et al.*, 2004). In pepper (*Capsicum annuum* L.) an increase in phenolic acids reduced lesion length and invasion upon infection by *Phytophthora capsici*. Susceptible cultivars of *C. annuum* produced lower amounts of phenolic acids and lesion lengths were not reduced. The compound with the most distinct inhibitory effect was *t*-cinnamic acid, followed by *p*-hydroxybenzoic-, vanillic- and salicylic acids (Candela *et al.*, 1995). Inhibition of growth of *Phytophthora megakarya* Bra. & Grif. in cacao (*Theobroma cacao* L.), which causes black pod disease, was also linked to the production of phenolic acids, especially apigenin and luteolin, but also other unidentified compounds (Djocgoue *et al.*, 2007). Although some variations have been reported with

regards to phenolic acids in the defense response, it appears that phenolic storing cells play a key role in the defense strategies of plants (Beckman, 2000). Cahill and McComb (1992) demonstrated that phenolic acid expression and lignin production in *E. calophylla* roots constitute the primary resistance mechanisms to *P. cinnamomi*. Phenolic compounds could perhaps play a dual role in *Eucalyptus* plants by acting as precursors for increased lignin synthesis, and as newly synthesised compounds which could act as fungitoxic substances (Cahill & McComb, 1992). Bekker *et al.* (2006) stated that silicon application to avocado has a direct mechanism of action as well as an indirect action on *P. cinnamomi* by inducing the trees to secrete phenolic compounds (Bekker *et al.*, 2006). Phenolic compounds as well as other fungitoxic substances were confined to the plant parts that received silicon applications in the form of potassium silicate. Heightened resistance in avocados to *P. cinnamomi* by silicon application, is likely a result of increased phenolic levels (Bekker *et al.*, 2007).

The HR is a well-known phenomenon in plants against pathogen attack. Many pathways interact in complex ways during the HR. Early responses include the oxidative burst, which upregulates the expression of various molecules such as ROS (hydrogen peroxide and superoxide molecules), peroxidases, etc. (Agrios, 2005). There are however controversial theories regarding the role of ROS and the HR in defense against *P. cinnamomi*. In *Arabidopsis thaliana* L., it has been shown that an increase in ROS production occurred after being challenged by *P. cinnamomi* zoospores as soon as 6 h post infection (Rookes *et al.*, 2008). On the other hand, the HR can also cause cell death, which was proposed to facilitate an increase in *P. cinnamomi* growth in susceptible avocado roots (García-Pineda *et al.*, 2010).

Phytoanticipins are antimicrobial low molecular weight compounds produced by plants, which are part of the plant's normal development and are present prior to exposure by microorganisms or formed by compounds that are formed by preexisting constituents after antimicrobial exposure. "Phytoalexin", on the other hand, is a collective term for various molecules that act like antibodies, but have very low specificity (VanEtten *et al.*, 1994). As a

general rule, phytoalexins are more toxic to fungi than to bacteria. Glyceolins are found in soybean roots, pisatin in pea pods, phaseolin in bean pods, sweet potato roots contain ipomeamarone, and trifolirhizin has been described in red clover roots. In mostly dicots, more than 150 phytoalexins have been identified (Salisbury & Ross, 1992). Ham *et al* (1991) proposed that β -1,3-glucanase in soybeans releases a phytoanticipin elicitor from *Phytophthora megasperma* cell walls, which causes the release of a phytoanticipin, which in turn plays a role in disease resistance against this oomycete pathogen.

The biochemical makeup of plant roots can also influence the growth of pathogens *in vivo*. In a study by Sánchez-Pérez *et al.* (2009) it was demonstrated that crude root extracts of certain avocado rootstocks could almost completely inhibit the growth of *P. cinnamomi*. In *Persea borbonia* L., which is resistant to *P. cinnamomi*, a preformed biochemical mixture was isolated from the stems as well as the roots. This antifungal mix consisted of four similar compounds, of which the most prominent was identical to a previously described compound named isoobtusilactone A. The name “borbonol” was given to the mixture, and it inhibited vegetative growth as well as sporangia formation *in vitro* at relatively low concentrations (Zaki *et al.*, 1980). This study by Zaki *et al.* (1980) also confirmed the presence of borbonol in low amounts in the stems and branches of several *P. americana* cultivars including ‘Duke 7’, but also in the susceptible rootstock ‘Topa Topa’; thus, the exact role of borbonol in PRR resistance is unclear. It is possible that borbonol is only expressed in older parts of the tree, leaving the younger roots still susceptible.

The cell walls of fungi contain chitin, but those of oomycetes consist mainly of cellulose and other glucans. Current PRR control practices include mulching, whereby the system relies on natural soil inhabiting microorganisms to decompose the cellulose components of plant material. In the process, hyphae of *P. cinnamomi* are also degraded (Downer *et al.*, 2001). Bartnicki-Garcia (1966) conducted foundational research by determining the cell wall composition of *Phytophthora* species (*P. cinnamomi* and *P. parasitica*). The study revealed that the cell walls contained about 90% glucans and a maximum of 25% cellulose II (a stable

form of cellulose). The glucans found in *P. cinnamomi* cytoplasm were in a later study determined to be mainly β -1,3- and β -1,6- linked glucans. Although no β -1,4-glucan digestion could be detected in the cytoplasm, the cell walls of *P. cinnamomi* seemed to consist of this type of glucan (Zevenhuizen & Bartnicki-Garcia, 1970). Several enzymes found in mulch systems break down these compounds in the cell walls of *Phytophthora* species and influence the fate of propagules of *P. cinnamomi*. Mulches consisting of wood chips, were found to contain predominantly wood-decaying fungi which produce exogenous cellulase enzymes (Downer *et al.*, 2002). In a study by Downer *et al.* (2001) the cellulytic enzyme cellulase (β -1,4-glucanase) was shown to inhibit the development of zoosporangia, chlamydospores and zoospores of *P. cinnamomi*. Although laminarase (β -1,3-glucanase) did not have a significant effect, both cellulase and laminarase significantly reduced the encystment of zoospores.

Molecules that consist of a β -1,4- glucan backbone can be broken down by β -1,4-glucanases. Avocado plants are able to produce β -1,4- glucanases, which are also called EGases. In tomato (*Lycopersicon esculentum* Mill.) EGases were expressed in etiolated hypocotyls at higher levels after treatment with ethylene as well as with auxin (Brummell *et al.*, 1997). Also, rapidly expanding tissues expressed higher EGase levels. Avocado fruit has been shown to contain endo- β -(1,4)-D-glucanases, which are regulated by a small gene family (Tucker & Milligan, 1991). This family of glucanases, if expressed in the roots of avocado, could enhance resistance against PRR. This mechanism of biochemical defense would allow the glucans in the cell wall and cytoplasm of the pathogen mycelium to be degraded. The observations of Ben-Ya'acov and Michelson (1995) that PRR tolerance in avocado was influenced by the rate of production of new healthy feeder roots may corroborate the hypothesis that such rootstocks also produce higher quantities of β -1,4-glucanases. Commercial avocado rootstock production now involves an etiolation step as well as auxin treatment for the stimulation of root development. Thus, higher glucanase production might also occur in the latter situations as well (Brummell *et al.*, 1997).

Rootstocks have been shown to affect the resistance of 'Hass' scions to postharvest anthracnose (Willingham *et al.*, 2001). Antifungal diene and mineral nutrients most likely

accumulate in the scion of the tree. It is possible that a rootstock could influence the biochemical expression of enzymes involved in the breakdown or synthesis of diene in the scion (Willingham *et al.*, 2001). This suggests that the presence of a water-soluble biochemical molecule that is presumably transported through the xylem is responsible for both resistance against root rot as well as against fruit pathogens. A study by Bower and Nel (1981) linked the rootstock-scion combination with peroxidase expression in the leaves, and also linked its expression to PRR resistance. Older trees were more vulnerable than younger trees, which expressed more peroxidase enzyme (Bower & Nel, 1981).

Discussion and Conclusion

Worldwide, the avocado is an economically important crop. Control of PRR is crucial for successful avocado production. Phosphite has both a direct and indirect mode of action against *Phytophthora* spp. (Smillie *et al.*, 1989). Thus far, phosphonate trunk injections have provided reasonable control (Pegg *et al.*, 1985; Pegg *et al.*, 1987), but resistance to this fungicide may increase with the current extensive use. *Phytophthora cinnamomi* is the most important biological threat to the avocado industry. Strict regulations regarding chemical usage intensify the need for developing PRR resistant rootstocks. Recently, reduced sensitivity to phosphite in some *P. cinnamomi* strains has been reported in Australia (Dobrowolski *et al.*, 2008). Although control is still adequate, the concern of complete resistance puts the industry under tremendous pressure, increasing the demand for research on resistance in rootstocks.

Although anatomical studies on various avocado cultivars have been conducted, research to assess resistance mechanisms in new rootstocks is needed. Histological changes have been observed in various hosts, including avocado, after being challenged with *P. cinnamomi*. Vacuoles, which were suggested to play secretory functions can play a role in defense. Other

cellular changes such as thickened cell walls and tyloses have also been observed, and may influence the compatibility between avocado and the pathogen.

Defense response pathways occasionally rely on specific biochemical compounds. These compounds could contribute to the tolerance observed in newly selected avocado rootstocks, and include phenolic compounds, ROS, hydrogen peroxide, superoxide dismutase, catalase, peroxidases and glucanases.

Mechanisms of resistance may be very pathogen-specific, whereas others utilize broad resistance mechanisms. Since avocado and *P. cinnamomi* did not co-evolve (Whiley *et al.*, 2002), resistance against PRR is probably non-specific. Nevertheless, the work of Phillips *et al.* (1987) suggests an active defense pathway, which contributes to resistance against *P. cinnamomi* in avocado plants. Whether the latest rootstock selections react as does the commercial standard 'Duke 7' remains to be determined. Currently, no truly PRR-resistant rootstock is available and the most tolerant rootstocks succumb under high disease pressure when no other control measures are used.

In this study, the infection process of *P. cinnamomi* will be investigated on a newly selected rootstock (R0.06), and compared to more susceptible rootstocks R0.10 and R0.12, by specifically focusing on the pre-penetration and germination of zoospores, as well as histological changes that might occur upon infection. Phenolic acid content, glucanase, catalase, superoxide dismutase as well as peroxidase expression will also be investigated and compared between rootstocks after avocado plantlets have been challenged with *P. cinnamomi* at various time intervals. This study will enhance understandings of the interaction between avocado and *P. cinnamomi*. Specific knowledge on the biochemical and cellular mechanisms of resistance could facilitate screening of rootstocks for resistance to PRR.

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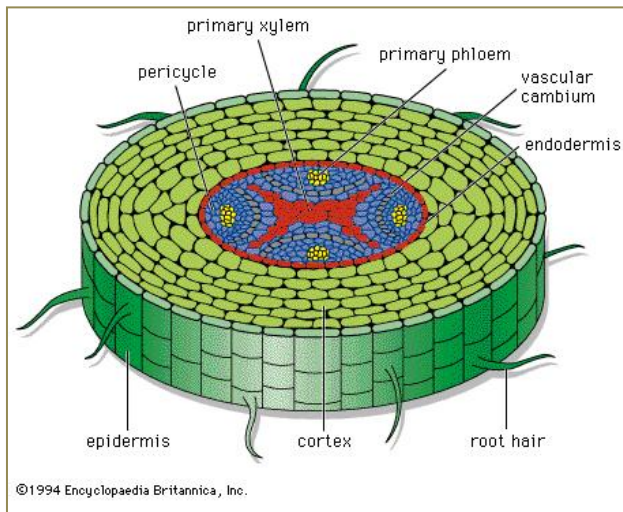


Figure 1.1. General root anatomy of a dicotyledonous plant (Microsoft Encarta, 2006)

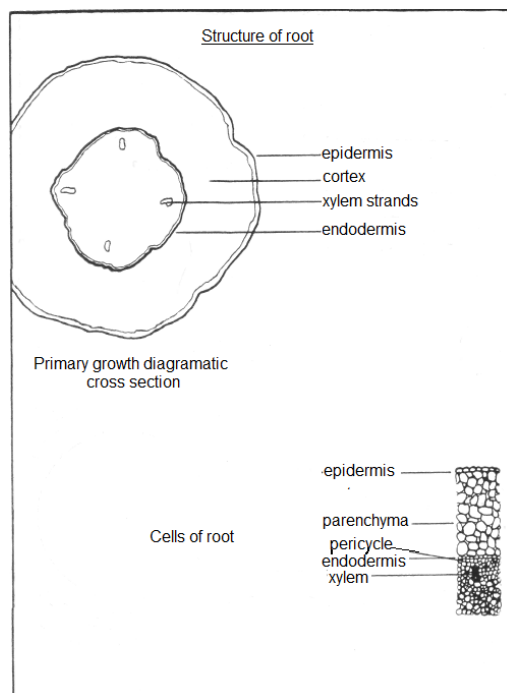


Figure 1.2. Diagram of a cross-section of an avocado root (Heismann, 1939)

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Table 1.1. Avocado rootstocks commercially available in California and South Africa (Reproduced from Bender *et al.*, 2010). Ratings are relative to location.

Cultivar	Topa Topa	G-6	Duke 7	Thomas	G755A,B,C (Martin Grande)	Barr Duke	Toro Canyon	D9	Borchard	Dusa (Merensky 2)	Latas (Merensky 1)	Zentmyer (PP4)	Uzi (PP15)
Productivity "clean" soil	3	3	4	2	1	3	3	2.5	4	4	?	2	3.5
Productivity in PRR soil	1	3.5	3	3	2	3	3.5	3	2	4	4	5	5
Resistance to Pc	0	2	3	4.5	5	3.5	2.5	3.5	0.5	5	4.5	5	5
Horticultural race	Mexican	Mexican	Mexican	Mexican	Hybrid: <i>P. americana</i> x <i>P. shiedeana</i>	Mexican	Mexican	Mexican	Mexican	Mexican x Guata	Mexican x Guata	Mexican	Mexican

Legend: 0 = poor, 5 = best

Chapter 2

**A histopathological approach to investigate possible mechanisms of
resistance in avocado rootstocks against Phytophthora root rot, caused by
*Phytophthora cinnamomi***

Abstract

Phytophthora root rot (PRR) of avocado, caused by *Phytophthora cinnamomi*, is a serious disease which leads to significant global economic losses annually. Although the most preferred disease control method is the use of root rot-resistant plants, a completely resistant rootstock has not been developed. Poor understanding of resistance mechanisms against PRR impedes the development of such rootstocks. The objectives of this study were to microscopically distinguish between defense responses of different avocado rootstocks varying in PRR resistance, R0.06 (resistant), R0.10 (moderately resistant) and R0.12 (susceptible). Zoospore-inoculated avocado roots were harvested and fixed at various times after inoculation and the amount of cysts and their germination rates were quantified and compared by means of scanning electron microscopy. Fixed roots were also embedded in JB-4 resin, sectioned with a microtome and stained with Calcofluor and subjected to confocal microscopy. Although chemotaxis did not appear to play a role in resistance, inhibition of cyst germination was greater on R0.06 than R0.10 and R0.12. Furthermore, callose depositions formed as early as 6 hpi in resistant rootstocks, whereas lignification occurred in susceptible R0.12 roots. Four days after inoculation R0.12 xylem vessels were entirely occluded by tyloses. Callose depositions increased in both R0.06 and R0.10 root cortices, but lignification also occurred in R0.10 which compromised the amount of callose that formed such that R0.06 had more callose depositions at 12 dpi. Lignified cells became increasingly abundant in R0.12 after 12 days, but were ineffective against PRR since roots were completely colonised by *P. cinnamomi*. No tyloses were observed in R0.12 at 12 dpi. The resistant rootstock, R0.06, suppressed hyphal growth, and few or no hyphae were detected in roots after 12 days; more hyphae were detected at this time in R0.10, although not as profusely as in R0.12. Results from this investigation enhanced our understanding of the interaction between *P. cinnamomi* and avocado, and indicated which defense mechanisms may be needed in future rootstock selections.

Introduction

Phytophthora cinnamomi Rands causes Phytophthora root rot (PRR) of avocado (*Persea americana* Mill.), which results in major losses in production. Global avocado production has increased steadily over the past 50 years (FAOSTAT, 2009). In 2009, the global avocado industry produced more than five times the volume of that produced in 1961. However, this increase in global avocado production can primarily be attributed to an increase in total production area (FAOSTAT, 2009). Despite more than half a century's research, absolute control of PRR has not been achieved.

The most effective means of plant disease control is usually achieved through integrated disease management. Control of PRR of avocado can be achieved by the use of phosphorous acid trunk injections (Pegg *et al.*, 1987). Mulching of avocado orchards has become an essential cultural practice. Not only does mulching provide organic material that enhances feeder root health, but it increases populations of natural microorganisms. These microorganisms increase the amount of cellulolytic enzymes in mulched soils, which in turn, are detrimental to *P. cinnamomi* (Downer *et al.*, 2002; Richter *et al.*, 2011). Lastly, PRR tolerant rootstocks are becoming increasingly common in commercial production. However, only root rot tolerant (or partially resistant) rootstocks have been commercialised. Greater understandings of the mechanisms that are responsible for tolerance/resistance to PRR are needed to improve these rootstocks.

Genetic transformation creates opportunities for improving rootstock resistance. Transformation of perennial crops is challenging, but has had limited success in avocado (Ahmed *et al.*, 1997; Cruz-Hernández *et al.*, 1998; Litz *et al.*, 2007a,b; Pliego-Alfaro & Murashige, 1988). Furthermore, the possibility of genetically engineering avocado rootstocks to increase resistance by means of RNA silencing is currently being considered (Mitter *et al.*, 2011).

For the purposes of this study, tolerance is defined as the ability of a host plant to reduce the effect of a pathogen on plant fitness, health and yield; whereas resistance is the ability of a plant to reduce/inhibit/restrict pathogen colonisation by means of an active defense response that results in an increase in plant health. Susceptibility is referred to as the severity of disease symptoms that is caused by a pathogen (Agrios, 2005; Kover & Schaal, 2002; Manners, 1993).

Plants possess mechanisms to defend themselves against pathogen invasion that are categorised into two groups, namely biochemical and structural defenses. A broad range of general defense-related biochemicals are found in plants. For instance, reactive oxygen species (ROS) are generally effective against biotrophic pathogens. Other biochemical defense molecules include phenolic acids, phytoalexins (which are induced by glucans elicited from oomycete cell walls), and proteins produced by the ethylene, jasmonate and salicylic acid pathways (Agrios, 2005; Clarke *et al.*, 2000; García-Pineda *et al.*, 2010; Glazebrook, 2005; Yoshikawa *et al.*, 1981). Plants also possess resistance mechanisms which are very specific to certain pathogens; however, this is unlikely for the *P. cinnamomi* x *P. americana* interaction since these organisms are native to different continents and did not co-adapt (Whiley *et al.*, 2002). The second group of mechanisms is structural defenses, and include physical barriers such as tyloses, lignification and callose production.

The formation of tyloses is categorised as a histological defense response since it forms in advance of pathogen growth. Tyloses are outgrowths of parenchyma cells into xylem vessels, which block the flow of water to minimise stress, but also reduce pathogen advancement in vascular bundles (Manners, 1993). Tyloses can consist of various combinations of lignin, cellulose, and cross-linking glycans, as well as substances with a primarily cellulosic nature (Ek *et al.*, 2009; Williams, 1942). Various plant host responses have been associated with tylose formation after infection with *Phytophthora* spp. (Davison & Tay, 1987; Riedel *et al.*, 2009). In avocado, tylose formation has been observed in the roots of the moderately PRR-resistant rootstock 'Duke 7' after inoculation with *P.*

cinnamomi (Phillips *et al.*, 1987). Although tylose formation could yield plants resistant by reducing pathogen spread (Agrios, 2005), they also have an irreversible, negative impact on hydraulic conductivity (Neuhaus *et al.*, 2007).

Cell wall structures that play a role in pathogen defense include lignifications, suberisations, callose depositions and phenolic substances which are cross-linked with cellulosic materials (Agrios, 2005). Lignification of plant cell walls has been associated with disease resistance, including resistance to PRR (Cahill & McComb, 1992; Kuć, 1982; Vance & Sherwood, 1976). Callose papillae have also been associated with resistance in several plant species against *P. cinnamomi* (Cahill & Weste, 1983; Hinch & Clarke, 1982). Autofluorescent phenolic compounds, lignin, as well as material that stains with aniline blue increased in 'Duke 7' roots after infection with *P. cinnamomi* (Phillips *et al.*, 1987).

Apart from active resistance mechanisms, plants often appear to be resistant as a result of disease escape, hence the term "apparent resistance". Apparent resistance results whenever one or more of the three factors in the disease triangle (susceptible host, favourable environment and virulent pathogen) are absent or do not coincide to cause disease (Agrios, 2005). Disease-tolerant plants often produce roots at a faster rate than susceptible plants, ensuring that there is always a certain percentage of healthy, uninfected functional roots (Graham, 1995). Another such disease-escape phenomenon in the *P. cinnamomi*-host interaction is low excretion of root exudates by tolerant host plants that are responsible for zoospore attraction (Botha & Kotze, 1989), which is an important factor to consider when mining for resistance mechanisms in plants.

Zoospores are attracted to specific biochemicals by means of chemotaxis. *Phytophthora cinnamomi* is attracted to most amino acids, certain phenolic compounds, alcohols and isovaleraldehyde (Botha & Kotze, 1989; Cahill & Hardham, 1994). Zoospores are generally attracted to the elongation area, situated behind the root tip, which differ slightly among

plant species (Hinch & Weste, 1979). Several PRR-resistant plant species attract fewer zoospores. For instance, roots of resistant blueberry varieties attract fewer *P. cinnamomi* zoospores than susceptible varieties (Agrios, 2005). In avocado, more *P. cinnamomi* zoospores are attracted to roots of the susceptible cultivar, 'Edranol', than the more resistant rootstocks 'Duke 7', 'G6' and 'Martin Grande' (Aveling & Rijkenberg, 1991; Botha & Kotze, 1989). These observations are further supported by Zilberstein & Pinkas (1987) who demonstrated that 'Duke 7' and 'G6' excreted significantly fewer electrolytes than susceptible cultivars after *in vitro* inoculation.

Not only does the presence of a pathogen determine disease incidence, but its ability to establish, germinate and infect a plant also influences disease severity. For instance, fewer zoospores of *Aphanomyces euteiches* Drechs., an oomycete root rot pathogen, germinated on resistant pea cultivars than on susceptible cultivars (Kraft & Boge, 1996). Aveling & Rijkenberg (1991) demonstrated similar results in the *P. cinnamomi*-avocado interaction, where fewer zoospore cysts germinated on roots of more resistant cultivars than on susceptible 'Edranol' seedlings.

Despite several attempts to identify major mechanisms of resistance, the interaction between *P. cinnamomi* and *P. americana* is poorly understood. Furthermore, resistance mechanisms have not been studied in PRR resistant rootstocks selected during the past two decades. For instance, mechanisms of tolerance/resistance in the highly PRR resistant rootstock, Dusa[®], are currently unidentified. The histological examination performed by Phillips *et al.* (1987) provided valuable information regarding structural defense mechanisms utilised by avocado plants. However, since no comparison has been made between resistant and susceptible rootstocks, no conclusions can be formulated as to which resistance mechanisms are responsible for tolerance observed in the field. In addition, none of the described mechanisms for resistance are currently being considered in commercial rootstock selection programmes, possibly because defense against *P. cinnamomi* in plants is thought to be controlled by several genes (Cahill *et al.*, 1989).

The objectives of this study were to determine the role of zoospore behaviour on roots of three avocado rootstocks varying in resistance to PRR, and to make histopathological comparisons among these rootstocks to elucidate the role of structural barriers in defense against *P. cinnamomi*. We hypothesised that: i) fewer *P. cinnamomi* zoospores would be attracted to resistant than susceptible rootstocks; ii) inhibition of zoospore cyst germination would increase with an increase in rootstock resistance; and iii) structural barriers such as lignin and callose papillae would form in resistant rootstocks more rapidly and in greater abundance upon infection by *P. cinnamomi*. Results obtained from this investigation will illuminate differences in defense responses against *P. cinnamomi* within and among avocado rootstocks which vary in resistance. This will in turn provide a better understanding of essential defense mechanisms that may be needed in future rootstocks.

Materials and Methods

Production and maintenance of avocado plants

Three avocado rootstocks were used in this trial: R0.12 (susceptible), R0.10 (moderately resistant), and R0.06 (resistant). Although no avocado rootstock is fully resistant, R0.06 is referred to as “resistant” as it was the most resistant of the available rootstocks. Avocado plantlets were clonally propagated and kindly provided by Westfalia Technological Services in Tzaneen, Limpopo province, South Africa. Plants were grown in black plastic bags containing perlite growth medium [Chemserve Perlite (Pty) Ltd, Rieperpark, South Africa] and watered three times per week by means of a light mist spray. Individual plants were fertigated with 50 ml Hoagland’s solution (Hoagland & Arnon, 1950) every 2 weeks. Phytotron temperatures ranged from 25°C ± 2, relative humidity was approximately 40%, and diurnal 12h/12h light was used.

Root biomass comparisons

Roots of five plantlets of each rootstock were harvested and lyophilised using a VirTis

adVantage lyophiliser (SP Scientific, New York, USA). Total root dry weight of each plantlet was measured gravimetrically.

Isolates

Phytophthora cinnamomi was isolated from diseased avocado roots in Tzaneen, using PARPH selective medium [10 mg pimaricin, 250 mg ampicillin, 10 mg rifampicin, 100 mg pentachloronitrobenzene (Sigma-Aldrich, Steinheim, Germany) and 50 mg hymexazol (Tachigaren, Sankyo Company, Tokyo, Japan) in 1 L water agar]. Cultures were maintained in 15 ml glass bottles containing sterile distilled water and lawn grass. *Phytophthora cinnamomi* isolates were positively identified by means of PCR using LPV3 primers (Reverse 5'-GTCCAAACCGACTCTTGCTCGATG-3' and Forward 5'-GTGCAGACTGTCGATGTG-3'; Kong *et al.*, 2003). Mating types were determined by following a modification of the method of Tooley *et al.* (1989), by plating out known *P. cinnamomi* mating type strains opposite unknown isolates at 20°C on 20% V8 agar (V8 juice supernatant was prepared by centrifugation with 10 g/L CaCO₃ for 20 min at 2 000 g; 15 g agar/L). Isolates used varied between experiments according to the ability to produce zoospores.

Inoculation of plants and preparation of samples

Inoculum production, infection and harvesting

Phytophthora cinnamomi (A2 mating type) zoospores were produced by placing three 5 x 5 mm V8 agar blocks containing the inoculum in 90 mm Petri dishes before adding approximately 20-25 ml 2% V8 broth. Plates were incubated at 20°C for 4 days whereafter mycelia were rinsed three times with distilled water. After rinsing, 20 ml non-sterile, double-filtered (Whatman filter paper) stream water was added to each dish and incubated at 20°C under a fluorescent light (350 nm) for 24-48 hours. Zoospores were released 1 h after a 45 min cold shock at 4°C. Zoospore concentrations were determined with a haemocytometer after vigorous vortexing.

Inoculation procedure for determination of zoospore attraction differences among rootstocks

Roots of the rootstocks were inoculated *in vitro* with 3.5×10^4 ml⁻¹ zoospores of *P. cinnamomi*. Roots of each rootstock were placed in separate sections of a porous, compartmentalised Petri dish that contained the zoospore suspension. Roots were harvested at 1, 3 and 6 hpi, fixed in FAA [formalin, acetic acid, alcohol (95% ethanol) and water: 2:1:10:7], and stored at room temperature.

Inoculation procedure for comparing zoospore germination rate differences and histopathology among three rootstocks

Roots of the rootstocks were infected with 6×10^4 ml⁻¹ zoospores via dip-inoculation. Plantlets were kept moist by frequently applying a light water mist spray in a plastic bag for 1 h before planting in vermiculite. Roots were harvested at 0, 1, 3, 6, 12, 24, 48, 96 and 288 hpi and fixed in FAA as before.

Preparation of samples for Scanning Electron Microscopy (SEM)

Fixed roots, which were harvested at 1, 3 and 6 hpi, were washed three times for 15 min in 0.075 M sodium phosphate buffer (pH 7.4). Samples were dehydrated in an ascending ethanol series by incubation for 15 min in 30, 50, 70 and 90% ethanol. Specimens were then placed in 100% ethanol three times for 15 min, before drying to a critical dry point. Double-sided conductive carbon tape was used to mount samples on aluminium slides before sputter coating with gold (Emitech K550X, Quorum Technologies Ltd, Ashford, Kent, UK). Inoculated root samples were then visualized with a Jeol 840 scanning electron microscope (Tokyo, Japan).

Image processing and data collection

Scanning electron micrographs (80-100 X magnification) were used to construct whole root images by overlaying contiguous regions of images with Photostitch® software. Roots were divided into four regions, 2 mm apart (Figure 2.1; Zeiss Axiovision 4.7, Carl Zeiss Ltd., Munchen, Germany). Zeiss Axiovision 4.7 was also used to construct a 500 µm² square block, which was randomly placed in each of the four regions on the roots to count zoospore cysts and germinated cysts.

JB-4 plastic embedding

Samples obtained at 0, 3, 6, 12, 24, 48, 96 and 288 hpi were embedded in a JB-4 resin. Fixed roots were subjected to vacuum conditions for 15 min and incubated at room temperature for 2 h. Root samples were washed three times for 15 min in 0.075 M sodium phosphate buffer (pH 7.4) and subjected to an ethanol dehydration series (30, 50, 70, 95 and 100%) by incubation for 15 min in each concentration, followed by a final incubation in absolute ethanol for 1 h. Samples were further processed according to the manufacturer's instructions (JB-4), except that a final vacuum infiltration was performed for 15 min on all samples during the final incubation in an infiltration solution. Roots were embedded in BEEM® bottle neck tip capsules (EMS, Hatfield, PA), and all procedures were carried out on ice.

Sectioning and staining of samples

A Leica cryomicrotome (Leica, Nussloch, Germany) was used at room temperature to section JB-4 embedded samples. Manual modifications were performed as samples were mounted on 10 mm bolts using Pratley Quickset Clear® (Figure 2.2). Section thicknesses ranged from 10-20 µm and samples were immediately placed in distilled water droplets on slides, which in turn were placed on a heating block at 35°C. Dry slides containing root sections were stained with 0.01% calcofluor white fluorescent brightener 28 (Sigma-Aldrich, Steinheim, Germany) for 5 min and rinsed in tap water for 2 min. Cover slips were mounted

with Entellan® (EMS). Hand sections of selected fixed samples were also made and stained with calcofluor.

Morphological comparisons

Stereo microscopy

Root thickness was measured by stereo microscopy. Six whole feeder roots of each of five plantlets per rootstock (30 roots in total per rootstock) were placed on a Petri dish and observed under a Zeiss stemi 2000 stereo microscope (Carl Zeiss Ltd., Munchen, Germany). Digital microscopic photographs were obtained and root measurements determined with an HRc Axiocam digital camera and the accompanying Axiovision 4.7 software. Average diameter measurements were used to construct schematic representations using SolidWorks® 2008 software (Dassault Systemes SolidWorks Corp., 300 Baker Ave Suite 110, Concord, MA 01742).

Anatomical comparisons

Fluorescent light microscopy

Five noninoculated, JB-4-embedded, root samples of each rootstock were examined to compare their anatomy. Sections were visualised under blue UV light, using a Zeiss Axioskop microscope, but occasionally the autofluorescent filters (red and green light) were also used. Digital images were obtained as described above. Xylem vessel areas were determined using Zeiss LSM Image Browser Version 4.2.0.121 (Carl Zeiss Ltd.) from *.zvi files that were generated with Axiovision 4.7 software.

Histopathological comparisons

Confocal microscopy

Digital microscope images were obtained using a confocal laser scanning microscope (CLSM 510 Meta, Carl Zeiss Ltd.). Two wavelengths were used, 405 nm (BP 420-480) to fluoresce calcofluor white-stained materials, and 543 nm (LP 560) for autofluorescence. Images were processed using Zeiss LSM Image Browser Version 4.2.0.121 (Carl Zeiss Ltd.).

Selected samples were stained with 0.05% aniline blue instead of calcofluor white, and viewed under a fluorescent microscope (Zeiss Axioskop; Carl Zeiss Ltd.) The same materials that fluoresced after calcofluor white-staining appeared to fluoresce when stained with aniline blue. Aniline blue stains callose, but these materials contain both β -1,3- and β -1,4-glucan linkages (Cahill & Weste, 1983). Calcofluor white stains cellulose, callose and other glucan linkages (Cheng, 2006; Dardelle *et al.*, 2010). Therefore, the term “callose” is referred to as material that fluoresces brightly blue due to calcofluor-staining, although compounds other than callose were probably also visualised.

Statistical analysis

Data generated from the biomass, morphological and anatomical comparisons were subjected to an analysis of variance (ANOVA) using the General Linear Model Procedure from SAS software (Version 9.2) and Fisher's protected Pairwise Test (SAS Institute Inc. 2004).

Results

Root biomass

On plants that were the same age, the resistant R0.06 rootstock produced more roots than R0.10 and R0.12, although the difference between R0.06 and R0.10 was not significant (Figure 2.3). Root biomass of R0.06 was significantly more than that of the susceptible

R0.12. Moderately resistant R0.10 root biomass did not differ significantly from both R0.06 and R0.12.

Root morphology

Roots of the susceptible rootstock, R0.12, were thicker than the moderately resistant R0.10 and resistant R0.06 rootstocks. One millimetre from the root tip, there were no significant differences in diameter between the three rootstocks (Figure 2.4). From 5 mm and onwards, root diameters of R0.06 were significantly smaller than R0.12. No significant differences were detected at any distance from the tip between R0.10 and R0.06 or between R0.10 and R0.12, although there was a slight indication that there could be differences between the latter.

Infection process and pathogen invasion - Scanning Electron Microscopy (SEM)

i) Zoospore attraction

Phytophthora cinnamomi zoospore cysts were observed on root surfaces of all three rootstocks. Zoospores were usually attracted to the area behind root tips (Figure 2.5). Interestingly, in region 1, significantly more zoospores were attracted to the resistant R0.06 and moderately resistant R0.10 rootstocks than the susceptible R0.12 rootstock (Figure 2.6). In both R0.06 and R0.10, a significantly higher amount of cysts were present in region 1, compared to region 2, 3, and 4 where solitary cysts were observed. However, in R0.12, the amount of encysted zoospores did not differ significantly between region 1 and 2, and 1 and 3, which was an indication that zoospores were attracted to a larger area on R0.12.

ii) Germination rate

The germination rate of *P. cinnamomi* zoospore cysts on rootstocks was directly proportional to the susceptibility of respective rootstocks. Subsequently, the resistant R0.06 showed a greater capacity to inhibit cyst germination than both R0.10 and R0.12; likewise, cysts on R0.10 germinated slower than on the susceptible R0.12 roots (Figure 2.7). On R0.06

roots, the fraction of germinated cysts at 1 hpi was only 30%, and was significantly less than at 3 hpi. Similarly fewer germinated cysts were detected at 3 (45%) than at 6 hpi on the same rootstock. By 6 hpi, the percentage of cysts that had germinated was also significantly lower on the resistant R0.06 (65%) than on susceptible R0.12 (85%) roots. Contrarily, on R0.12, cyst germination had occurred at maximum level as soon as 1 hpi, since the germination rate did not differ significantly between 1, 3 and 6 hpi. On R0.10, the proportion of germinated cysts was significantly lower at 1 hpi, than at 3 hpi, however, at 3 hpi the percentage of germinated cysts did not differ from 6 hpi and the percentage of germinated cysts was comparable to R0.12 roots at both 3 and 6 hpi.

iii) Infection process - external

As mentioned, zoospores were mainly attracted to the elongation zone which is situated behind the root tip of all rootstocks. Apart from the fact that the germination potential of *P. cinnamomi* zoospore cysts was lower on resistant rootstocks, no differences in pathogen infection structures/strategies were detected on root surfaces between rootstocks. For this reason, differences between rootstocks were not compared, and only differences across time points are considered, with a focus on the pathogen's infection strategies and its progression over time.

One hour after inoculating roots with *P. cinnamomi*, not all cysts had germinated (Figure 2.8A). Several germinated cysts contained appressoria-like structures (Figure 2.8B). Hyphae were usually short, although numerous hyphae had progressed relatively far away from the germinated cysts. Direct penetration of hyphae was frequently observed and often appeared to be attracted to specific micro-regions (Figure 2.8C). Direct penetration often occurred in grooves between epidermal cells. However, there was no evidence of cellulolytic degradation before penetration. Indirect penetration frequently occurred through openings or cracks on the root surface. Bacteria were present on most root samples, however, bacteria were often localised to areas surrounding cysts, which appeared

to be detrimental to the pathogen, especially as cysts were often degraded when bacteria were present in abundance (Figure 2.8D).

Few differences were detected between 3 and 6 hpi with *P. cinnamomi* zoospores other than the fact that mycelia were more abundant at 6 hpi (Figure 2.9A). Most cysts had germinated and mycelia had grown to several times the length of those hyphae observed at 1 hpi. The percentage of appressoria formation did not appear to differ from the amount of appressoria observed earlier, however, more evidence of indirect penetration became apparent due to longer hyphae which were able to reach cracks or openings (Figures 2.9B & C). Appressoria were usually in close proximity to germinated cysts at 1 hpi, but at 3 and 6 hpi appressoria were formed further away from cysts (Figure 2.9D). At 6 hpi, intercalary appressoria were often visible (Figures 2.9E & F).

Anatomy and histopathology: Light - and confocal microscopy

Calcofluor fluorescent brightener 28 was useful to stain and fluoresce glucan linkages in the cell walls of plant cells as well as *P. cinnamomi* hyphae at 405 nm. The long pass wavelength from 543 nm was able to excite autofluorescent materials in roots from all samples in the red spectrum. The two wavelengths enabled the visualisation of different biochemical molecules in plant roots, although infrequent overlapping of fluorescence occurred. Fluorescent light microscopy was useful to view and compare relatively thin sections of avocado roots (Figure 2.10), but by using confocal microscopy, pictures could be taken at different focal distances to create z-stacks (Figure 2.11A), which in turn allowed for the construction of 3-dimensional images by means of virtual sectioning (Figure 2.11B).

Noninoculated control plants did not develop disease symptoms. No hyphae or zoospore cysts were detected in any of the roots investigated at 0 hpi. Autofluorescence in the red spectrum of most cells were observed, but epidermal cells usually fluoresced more brightly. Epidermal cells could often be distinguished from the exodermis (Figure 2.12A). Bright blue

fluorescence of calcofluor-stained material indicated that individual callose depositions were present in all three rootstocks. Cells in the cortex were uniform in shape, but not in size, as enlarged cells were randomly present in all three rootstocks. In distal tissue, approximately 3 mm from the root tip, cells appeared more irregular as a result of tissue differentiation. Granular depositions were observed in the red range in individual cortical cells, which appeared to be randomly dispersed (Figures 2.12B & C). An endodermis was often not well defined, but the stele was easily distinguished from the cortex and was marked by clusters of pericycle cells. Collateral vascular bundles were observed in all three rootstocks, i.e. phloem occurred on the abaxial side between xylem tissues (Figure 2.12D). Pericycle cells fluoresced brightly, usually did not completely encompass the stele, and were located on the abaxial side of phloem bundles in groups of cells. Tetrarch, pentarch, hexarch and heptarch xylem patterns were noted in all rootstocks. The average total xylem vessel area in the resistant R0.06 was significantly lower than in the susceptible R0.12. Total xylem vessel area in the moderately resistant R0.10 rootstock (Figure 2.12) did not differ significantly from R0.06 or R0.12 (Table 2.1). Autofluorescent structures in xylem tissue indicated the presence of tyloses, usually closer to proximal tissue in roots. These structures were occasionally observed in all three rootstocks, although not frequently. No clear anatomical difference between rootstocks was apparent at 0 hpi.

No apparent histological differences were observed between rootstocks at 3 hpi. *Phytophthora cinnamomi* was observed on the surface of all rootstocks, but in R0.12 both hyphae and haustoria-like structures were observed infrequently in, respectively, epidermal and exodermal cells (Figure 2.13A). No hyphae were detected in the cortex or stele of any rootstock. In several cases where hyphae had penetrated, a fissure was observed between the hyphae and plant cell walls (Figure 2.13B). As in control plants, tyloses were infrequently observed and usually not found on the acropetal side of a root. Figures 2.13C and D illustrate the presence of tyloses in xylem tissue of an R0.10 root. Nevertheless, most xylem vessels contained no tyloses. Numerous cells which contained autofluorescent granules were present in the distal adaxial cortex of R0.12 roots. No anatomical differences were

observed in R0.06 at 3 hpi compared to control plants, except for the presence of hyphae that were visible on all rootstocks' root surfaces.

Defense responses became apparent 6 hpi. Penetrating hyphae were observed in epidermal cells of the resistant R0.06 roots, but were usually accompanied by callose depositions encompassing the penetration sites in the abaxial cortex (Figure 2.14A). These thickened cell walls often occurred in wide areas alongside the epidermis of R0.06 roots. No histological changes were observed in the xylem or adaxial cortex of R0.06. Tyloses and cortical wall thickenings were infrequent in R0.10 roots 6 hpi (Figure 2.14B). In R0.12, lignification occurred in cortical cells as well as in epidermal cells at hyphal penetration sites (Figures 2.14C & D). Penetrated hyphae grew intercellularly, but did not appear to be affected by lignifications. No lignifications or site-specific callose depositions were observed in R0.10, as were observed in R0.12 and R0.06, respectively. Hyphae were often observed to penetrate the epidermis of all rootstocks. Deeper focal points often revealed that fissures were present below root surfaces (Figure 2.15).

At 12 hpi granules became increasingly abundant in R0.06 root cortical cells on the proximal side of the root. Granules were mostly restricted to the abaxial cortex (Figure 2.16A). The cortex contained individual cells with callose deposits along the cell wall. R0.10 and R0.12 had similar responses as tylose-formation increased in both, although most xylem vessels did not show this response. Lignification was also apparent in R0.10 and R0.12 rootstocks, as random cells in the cortex fluoresced brightly red (Figures 2.16B, C & D). Increased lignifications were absent in the resistant R0.06 roots. No hyphae were detected in the cortex in any rootstock.

In all R0.06 root samples viewed at 24 hpi, callose deposits were apparent, especially in tissue adjacent to the epidermis (Figures 2.17A & B). These wall thickenings were also present in abundance in random cells in the root cortex. Cortical cells near the root tip were

notably larger and thicker and exhibited a brilliant blue fluorescence. Development of tyloses was observed, although complete occlusion of individual xylem vessels occurred infrequently. No hyphae were observed within R0.06 roots at this time point. In R0.10 roots, random areas in the cortical cell walls contained callose depositions (Figure 2.17C) at 24 hpi; however, callose depositions did not completely surround cells. In proximal cortical cells, these wall thickenings were more abundant and nearly covered the majority of cells. Tylose development continued in R0.10, but usually did not fill all xylem vessels. Granules were abundant in the susceptible R0.12 distal root cortex cells. R0.12 had fewer callose deposits at 24 hpi in the cortex area than at earlier times, and compared to R0.06. Intracellular hyphae were observed in R0.12 root cortex cells (Figure 2.17D), and were also detected in the stele, although not abundantly.

Hyphae were detected for the first time at 48 hpi in R0.06 and R0.10 root cortical cells, compared to R0.12 where hyphae had progressed into the stele by 24 hpi. In R0.06, hyphae usually grew intercellularly (Figure 2.18A), and although intracellular hyphae were also detected, this was uncommon and usually accompanied by callose depositions around infected cells (Figure 2.18B). R0.10 roots also had increased callose depositions in abaxial cortical cells, as well as in exodermal cells (Figure 2.18C). Cell wall fortifications in central cortex areas were less apparent at 48 hpi than earlier sampling times. Hyphae were also visible inter- and intracellularly in R0.10 roots at 48 hpi, although intercellular hyphae were observed more frequently. Callose was infrequently observed in abaxial cortical cells of R0.12, and increased lignification was evident (Figure 2.18D). Hyphae in R0.12 grew more abundantly at 48 hpi, and had progressed to the adaxial cortex (Figure 2.18E). Although mycelia were further advanced at this time compared to 24 hpi, no hyphae were observed in vascular bundles, and hyphae usually grew intercellularly (Figure 2.18F).

Callose was abundant at 96 hpi in R0.06 roots, especially in the proximal cortex (Figure 2.19A). A significant accumulation of callose in abaxial cortical cells close to or next to epidermal cell layers occurred compared to earlier time points. Hyphae were scarce and not

easily detected, however, when present, hyphae mostly grew intercellularly. In an isolated case, hyphae were detected in a pericycle cell in R0.06, accompanied by the formation of callose papillae around the hyphae in the vessel (Figure 2.19B). Tyloses composed of lignin were also observed in R0.06 root xylem. However, xylem occlusions were atypical in R0.06 and were usually confined to single vessels. In R0.10, mycelia had progressed into the stele, but most hyphae were restricted to the abaxial cortex and epidermis. Individual callose papillae were present in the cortex, but mainly in the abaxial cortex next to the epidermis (Figure 2.19C). The autofluorescence signal was stronger in R0.10 at 96 hpi than earlier, indicating the presence of higher lignin content. Lignification in R0.06 was not observed. In R0.10, autofluorescent granules were occasionally observed. R0.12 roots had notably more lignin content in axial parenchyma, within the endodermis region and cortex areas by 96 hpi (Figures 2.19D, E & F). Xylem vessels of this susceptible rootstock were severely occluded by tyloses (Figures 2.19E & F). Hyphae were detected within lignified tissue in the axial parenchyma, as well as in cortical tissue. Hyphae were also abundant in the abaxial cortex and epidermal cell layers. Callose was observed in cortical tissue (Figure 2.19D), but these wall thickenings were sparse in R0.12 roots and occurred less frequently than in R0.06 and R0.10. Granules were also observed in R0.12 at 96 hpi and were located in adaxial cortical cells, and not often in the abaxial cortex.

Twelve days post inoculation (dpi), R0.06 contained fewer hyphae in roots than the moderately resistant and susceptible rootstocks R0.10 and R0.12, respectively. Abundant callose depositions were present in R0.06 root cortices, either by layers of cells that walled off infected tissue (Figure 2.20A) or individual cortical cells (Figure 2.20B). Hyphae were not easily detected in R0.06 roots; however, when present they were usually in the absence of callose (Figure 2.20C). Tyloses were occasionally noticed in individual xylem vessels, but did not differ from control plants in R0.06. In R0.10, hyphae were slightly more abundant than in R0.06, but were often difficult to detect. Hyphae grew inter- and intracellularly, and had progressed towards the adaxial cortex and stele (Figure 2.20D). R0.10 roots had a considerable amount of callose accumulations in abaxial cortical and exodermal cells (Figures 2.20D & F). Tyloses were frequently observed in individual xylem vessels, but never

encompassed entire xylem rays. Penetration by hyphae through fissures still occurred, as noted earlier (Figures 2.20E & F). In contrast to the more resistant rootstocks, R0.12 roots were entirely colonised by hyphae. Hyphae mostly grew in the root cortex, but were also present in the stele (Figures 2.20G & H). Hyphae also grew profusely in lignified axial parenchyma. Interestingly, no tyloses were observed in R0.12 roots at 12 dpi. Callose deposits and granular structures were infrequent in the cortex of R0.12 at 12 dpi.

Discussion

This is apparently the first comparative histopathological study between different avocado rootstocks after inoculation with *P. cinnamomi*. The most significant finding in this investigation was that *P. cinnamomi* infection was inhibited in the resistant R0.06 rootstock relative to that observed in the more susceptible rootstocks, R0.10 and R0.12. Inhibition of pathogen growth in the resistant rootstock occurred in two ways. Firstly, fewer *P. cinnamomi* zoospore cysts germinated on the resistant R0.06 rootstock compared to R0.10 and R0.12. Secondly, inhibition of *P. cinnamomi* hyphal growth occurred internally and was associated with callose production. Lignification occurred in the susceptible rootstocks, but appeared to be unsuccessful in countering growth of the root rot pathogen. These results provide useful insights into understanding defense mechanisms in avocado against *P. cinnamomi* infection.

Among the three rootstocks examined, R0.06 had a greater capacity to produce more root biomass, it had thinner roots but had smaller xylem areas than the susceptible R0.12 rootstock. The moderately resistant R0.10 rootstock did not differ from R0.06 or R0.12 in any of the abovementioned features. These results suggest that there is a direct correlation between root diameter and xylem vessel area. Fassio *et al.* (2009) conducted experiments to correlate sap flow in various combinations of grafted avocado rootstocks with xylem anatomy. They observed higher sap flow rates in 'Hass' when grafted onto rootstocks with higher xylem vessel areas. Under dry conditions and when managing soil moisture content to

control PRR, rootstocks with smaller xylem vessel areas might be beneficial. Germination of *P. cinnamomi* zoospore cysts occurred at a significantly lower rate on R0.06 than on R0.10 and R0.12. Aveling & Rijkenberg (1991) reported fewer germinated *P. cinnamomi* cysts on roots of the highly tolerant/resistant 'Martin Grande' rootstock than on more susceptible 'Duke 7' and 'Edranol', 4 hpi. In the present study, earlier as well as later sampling times were included, and it was demonstrated that cysts that had not germinated by 3 hpi had the potential to germinate by 6 hpi. At 6 hpi, the percentage of germinated cysts on R0.06 roots was significantly lower than on susceptible R0.12 roots, but did not differ from R0.10. Downer *et al.* (2001) demonstrated that increases in laminarinase (β -1,3-glucanase) concentration reduced the survival of *P. cinnamomi* zoospores. This result, together with results from chapter 3, correlates with the fact that the resistant R0.06 rootstock has a rapid increase in laminarinase production during the first 12 hpi. Whether laminarinase is excreted from avocado roots needs to be confirmed. The inhibition of cyst germination rate probably does not confer resistance, but the slight time-delay might provide extra time for the plants to respond rapidly prior to infection.

The attraction of *P. cinnamomi* zoospores did not differ significantly between R0.06 and R0.10. However, surprisingly fewer cysts were found on the susceptible R0.12 rootstock. It is noteworthy to mention that R0.12 was initially selected as a potential tolerant/resistant rootstock, but was later rejected due to its poor performance in field trials, indicating that this rootstock possesses some resistance. In a previous study, no significant difference was found between the amount of zoospores encysted on the moderately resistant cultivar 'Duke 7 and susceptible 'Edranol' roots (Aveling & Rijkenberg, 1991). Hinch & Weste (1979) compared the behaviour of *P. cinnamomi* zoospores on roots of 23 different plant species varying in resistance, and found no differences between the amount of zoospores attracted to susceptible and resistant species. Results from the present investigation thus affirms that the amount of zoospore cysts attracted to roots are not correlated with resistance. Botha & Kotze (1989) found that 'Edranol' seedling roots excreted more amino acids than 'Duke 7' and 'Martin Grande', and that these amino acids were responsible for the attraction of *P. cinnamomi* zoospores to roots. Thus, although the amount of cysts present on a specific

plant root is not correlated with resistance, the fact that fewer amino acids are excreted might indicate that resistant plants have more efficient utilisation of amino acids, e.g. protein synthesis. Nevertheless, the more resistant rootstocks attracted more zoospores in this study and the amount of amino acids that are excreted needs to be confirmed.

A rapid defense response was observed in R0.06 by means of callose production. Callose depositions initially occurred within 6 hpi alongside the adaxial side of the epidermis, but later became evident in the rest of the cortex. Deposition of callose is a common phenomenon in defense responses in plants. Cahill *et al.* (1983) investigated the formation of callose in several plant species in response to *P. cinnamomi* infection. They noted that callose was not produced in any of the susceptible species investigated, but did form in most of the resistant species. In maize, callose production in the abaxial cortex was detected 4 hpi and associated with resistance against *P. cinnamomi*. In the present study, callose depositions probably formed earlier than 6 hpi, but the exact time is unclear due to sampling limitations. Callose was also detected in the pericycle where callose papillae formed around hyphae.

A defense response also occurred at 6 hpi in the susceptible R0.12 rootstock. Although callose depositions were observed in R0.12 (usually in the cortex), these responses were not specific and did not appear to differ from control plants. However, while R0.06 and R0.10 produced callose in response to infection, the susceptible R0.12 produced lignin. Lignified exodermal tissues were observed in an apparent attempt to wall off infection at 6 hpi. In addition, R0.12 roots also produced lignified cells in the root cortex. Lignification of susceptible R0.12 root cortices increased over time. At 96 hpi high autofluorescence was detected in axial parenchyma, indicating the presence of abundant lignin content. However, an increase in hyphal growth was also evident in the susceptible rootstock, despite the increase in lignin content. These observations are in agreement with the work of Cahill *et al.* (1989), who compared histological responses of various plant species ranging from fully susceptible to fully resistant. They noted that rapid lignification of cell walls was completely

ineffective against infection by *P. cinnamomi*. However, in a later study by Cahill & McComb (1992), it was concluded that lignin and phenolic acid synthesis played a major role in *Eucalyptus* defense against *P. cinnamomi*. Nevertheless, results obtained in the present study suggest that lignin synthesis in avocado roots is not a successful defensive barrier to *P. cinnamomi*.

The moderately resistant avocado rootstock, R0.10, also showed the ability to produce callose in response to infection with *P. cinnamomi*. Although responses in this rootstock appeared to be effective to some extent, the distribution as well as abundance of callose was less specific than in the resistant R0.06 rootstock, since hyphae were detected more frequently in R0.10. Furthermore, R0.10 showed the ability to produce more lignin than R0.06; however, the amount of lignin as indicated by autofluorescence was not comparable to the vast amounts of lignin detected in susceptible R0.12 roots. It has already been noted that lignin formation seems to be ineffective against *P. cinnamomi*. There are different defense mechanisms in the three rootstocks: resistant rootstocks produce more callose and less lignin, whereas susceptibility is associated with the synthesis of more lignin with less callose.

Roots of R0.12 were severely occluded by tyloses 96 hpi. Williams (1942) conducted experiments to determine the composition of tyloses in oak trees (*Quercus* spp.), and found that tyloses can consist of various combinations of lignin and cellulose, depending on the species. Through hydrolysis with sulphuric acid, it was established that some tyloses consist of substances primarily of cellulosic nature. Sulphuric acid can also hydrolyse laminarin (Peat *et al.*, 1958), and the exact glucan-linkage composition was therefore not determined in the study of Williams (1942). Tyloses autofluoresced in the red spectrum and it is therefore assumed that avocado root tyloses consist primarily of lignin (Cheng, 2006). However, xylem occlusions could also consist of phenolic substances (Kitin *et al.*, 2010), and increases in phenolic content has been associated with increased tylose formation (Gonzalez *et al.*, 2001). Xylem vessels were not severely occluded in R0.06 and R0.10. Wilting symptoms

started to appear in R0.12 plants approximately 1 week after inoculation. García-Pineda *et al.* (2010) demonstrated that seedlings of a PRR-susceptible avocado cultivar showed wilting symptoms 4 dpi with *P. cinnamomi* mycelia. Present results therefore suggest that severe damage to the conductive system occurs several days before aboveground symptoms appear.

Interestingly, no tyloses were observed in the susceptible R0.12 rootstock 12 dpi. Hyphae grew profusely at this time and occurred throughout the cortex as well as in the axial parenchyma, pericycle, phloem and xylem. Collins *et al.* (2009) found similar results when they studied the amount of tylose formation in tanoak trunks infected by *Phytophthora ramorum* Werres, de Cock, & In't Veld. They observed a reduction in tylose formation in infected trees 7 weeks post inoculation. One hypothesis was that *P. ramorum* produces elicitors which degrade tyloses, since tyloses are essentially part of living host tissue. Moreover, tyloses contain thin cell walls which are presumably easily degraded. They associated the reduction in tyloses with a significant simultaneous increase in *P. ramorum* hyphae (Collins *et al.*, 2009). It is therefore possible that tyloses were degraded in the present study.

Penetration of epidermal cells by *P. cinnamomi* hyphae often took place through fissures. Results obtained by SEM showed that hyphae often grew into cracks or fissures on root surfaces. These cracks appeared to aid in adhesion and to be superficial, however careful examination of confocal images revealed that fissures often extended into the exodermis or occasionally into the cortex. It is possible that the fissures observed by SEM and confocal microscopy are similar in nature, but the possibility of cell wall degrading enzymes cannot be ruled out. In fact, this study illustrated that *P. cinnamomi* hyphae penetrated root epidermal cells through gaps.

Granules were often observed in cells of all rootstocks studied. Coarse as well as finer cellular inclusions were often visible, however their identity is not clear. Cahill *et al.* (1989) found similar inclusions in *Eucalyptus marginata* Sm. root cells after staining with toluidine

blue, and suggested with uncertainty that these granules were phenolic materials. Phillips *et al.* (1987) found cellular inclusions in avocado plants, also after toluidine blue staining, but referred to them as tannins. Tannins are polyphenolic molecules, which, just as other phenolics, have autofluorescent properties (Cox *et al.*, 2005). Phenolic compounds have been shown to have structures similar to some of the autofluorescent granules observed in this study (Hutzler *et al.*, 1998), but the autofluorescent properties of starch granules can also not be neglected. Several of the autofluorescent inclusions which were observed in this study resembled plasmodesmata, as described by Fitzgibbon *et al.* (2010) who obtained confocal images at 591 nm. However, since it is presumed that plasmodesma collars consist of callose, we will assume that the granular inclusions we observed with a LP 543 nm laser are not plasmodesmata, and possibly of phenolic nature. Beckman (2000) demonstrated that certain, randomly scattered specialised cells in the cortex contain phenolic compounds, which is consistent with the current observations. At this time, the functions of these inclusions are not clear.

With respect to defense against *P. cinnamomi*, it is thus concluded that resources in avocado roots are more efficiently utilised when callose, rather than lignin, is produced. One possible explanation for the increased effectiveness of callose is that it consists of β -1,3-glucan linkages, which are degraded by β -1,3-glucanases. *Phytophthora cinnamomi* hyphae consist of various glucan linkages, including β -1,3-glucan (Bartnicki-Garcia, 1966). It would therefore be detrimental to the pathogen to produce β -1,3-glucanase to degrade callose depositions, since this enzyme would degrade its own cell wall. The presence of abundant callose depositions in R0.06 and R0.10 at 96 hpi and 12 dpi, in contrast to limited callose depositions at these times in the susceptible R0.12, is strong evidence for this hypothesis. Although the early timing and high amount of callose depositions was associated with disease tolerance in avocado roots, this response might not result in complete resistance. Cahill & Weste (1983) concluded that although callose papillae was associated with defense in maize, it only appeared to contribute to tolerance by slowing down pathogen growth, and that additional factors are likely to be associated with resistance. Thus, callose production

may contribute greatly to resistance to PRR, but biochemical defences, such as ROS, would probably also contribute to resistance.

Conclusion

This study shed light on the infection strategies utilised by *P. cinnamomi* to infect avocado roots, but also investigated the host response to infection by documenting histological defense mechanisms in susceptible and resistant rootstocks. The resistant R0.06 rootstock was able to suppress and delay the germination of *P. cinnamomi* zoospores on root surfaces to a significantly higher extent than the moderately resistant and susceptible R0.10 and R0.12 rootstocks, respectively. *Phytophthora cinnamomi* infects plants in diverse ways: By indirect penetration through fissures on roots or by penetrating between epidermal cells (which is possibly accompanied by enzymatic digestion of host cells); or through the formation of appressorium-like structures. Root rot-resistant avocado rootstocks were able to respond rapidly to this invasion by the production of callose near the penetration sites. An increase in callose depositions 6 hpi developed in the cortex. In contrast to R0.06 and R0.10, susceptible R0.12 roots accumulated lignin in response to infection, which proved to be ineffective in restricting *P. cinnamomi* hyphae. Twelve days post inoculation, *P. cinnamomi* grew profusely through susceptible R0.12 roots. On the contrary, R0.06 and R0.10 roots contained limited hyphae at this time and whenever hyphae were observed, it was usually in the absence of callose depositions. Tylose development increased in the susceptible rootstock so that xylem vessels were almost completely occluded 4 dpi. Although tyloses can function as defense structures, they also obstruct the flow of water and cause wilting. Interestingly, no tyloses were detected at 12 dpi, which might reflect degradation of these structures by the pathogen. Results obtained in this study provided new insights into the complex interaction between *P. cinnamomi* and avocado roots. In addition to the knowledge gained on the host-pathogen interaction, the application of epifluorescence microscopy (especially confocal microscopy) was a useful tool and could be used for screening and selecting new PRR-resistant rootstocks.

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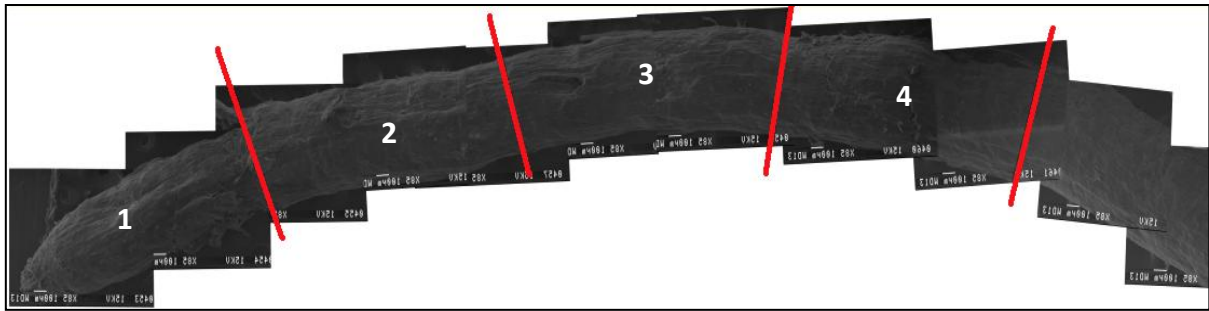


Figure 2.1. Construction of an avocado root image with overlapping regions of scanning electron microscope photographs and Photostitch® software. Four arbitrary regions, 2 mm apart, are indicated by numbers 1-4, with region 1 being closest to the root tip.



Figure 2.2. JB-4 embedded avocado root, mounted on a 10 mm steel bolt.

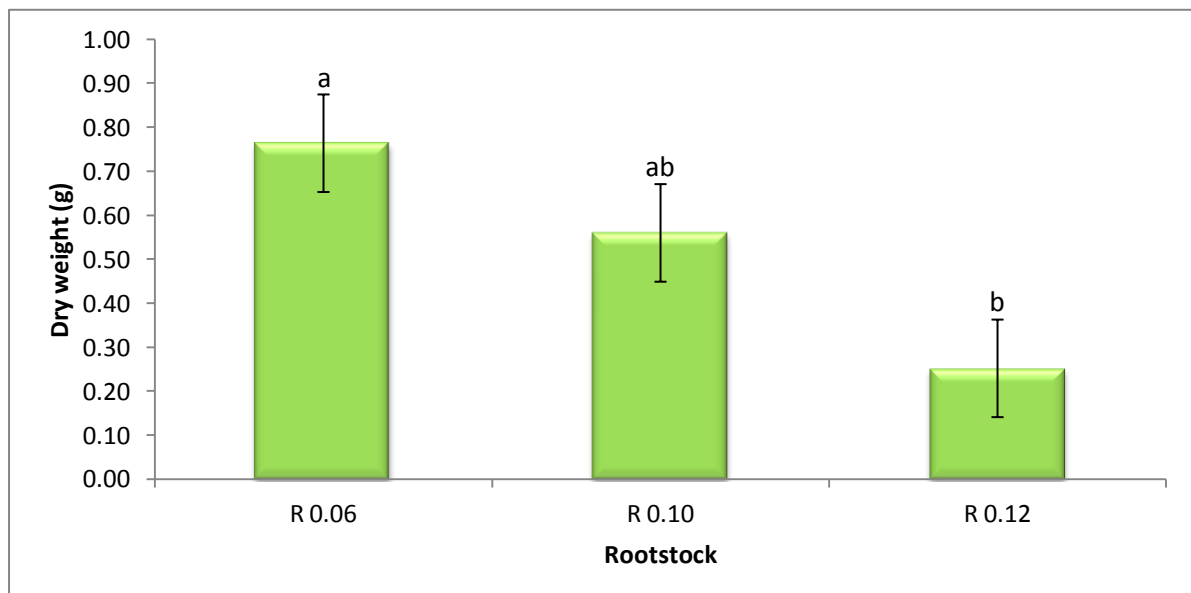


Figure 2.3. Root biomass (dry weight) of three 8 month-old avocado rootstocks. Bars are means of five randomly selected plants \pm SE ($\alpha=0.05$).

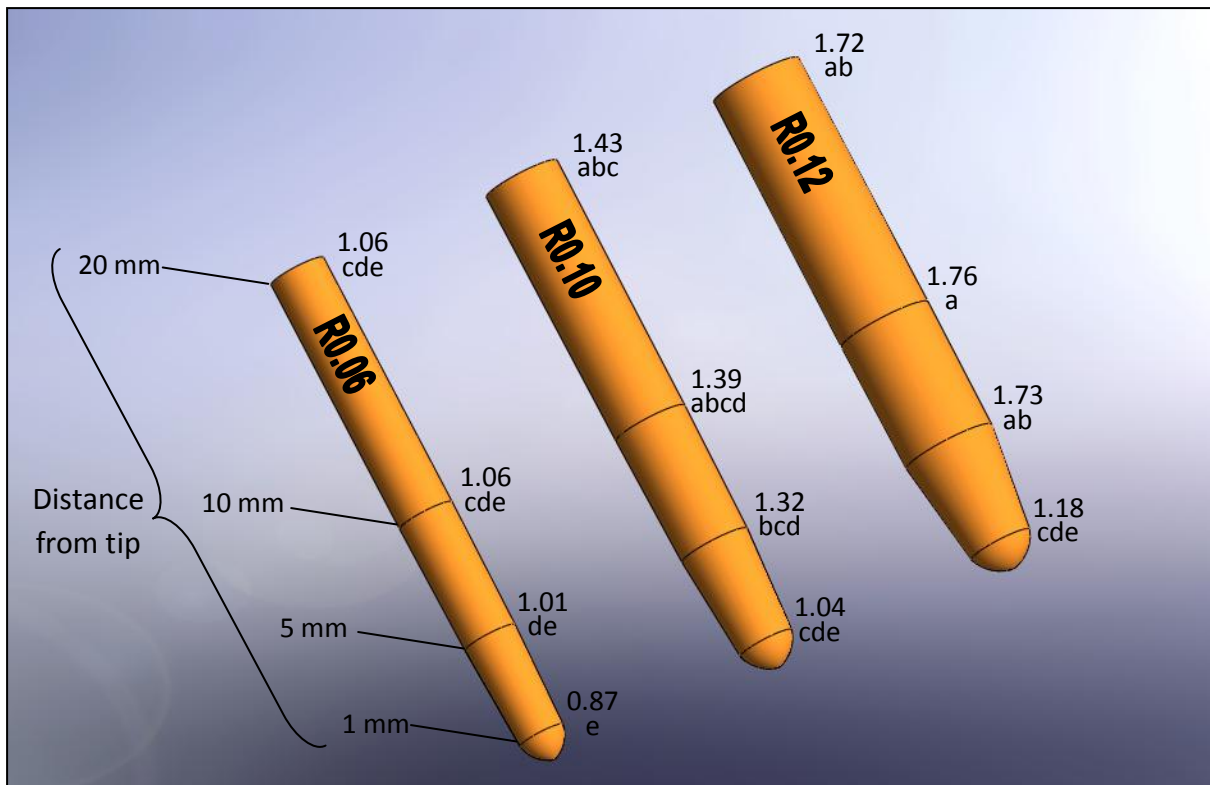


Figure 2.4. Schematic representations of root thickness for the R0.06 (resistant), R0.10 (moderately resistant) and R0.12 (susceptible) rootstocks. Value units are in millimetres and are indicated at distances 1, 5, 10 and 20 mm from root tips. Values are means of at least 30 samples (five plants with six replicates per rootstock). Same letters indicate no significant difference ($\alpha=0.05$).

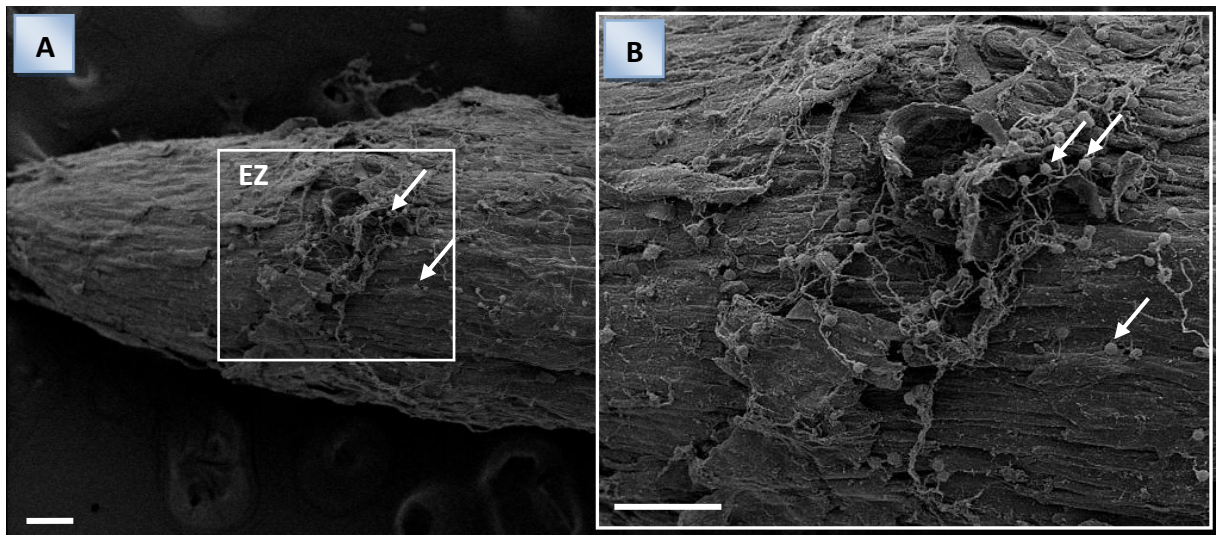


Figure 2.5. Scanning electron micrographs of a resistant R0.06 avocado root, 6 h after inoculation with *Phytophthora cinnamomi* zoospores. **A.** Zoospore cysts (arrows) are mostly confined to the elongation zone (EZ; square). **B.** Inset of **A.** Scale bar = 100 μ m.

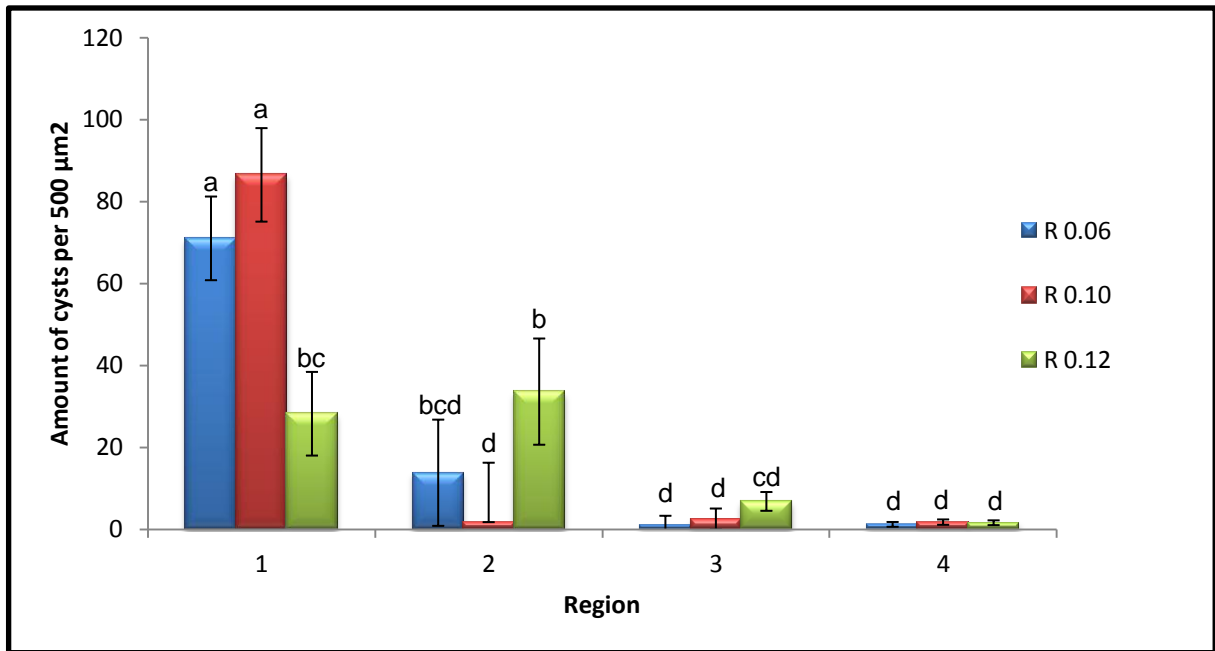


Figure 2.6. Amount of *Phytophthora cinnamomi* zoospores attracted to roots of three avocado rootstocks varying in resistance, namely R0.06 – resistant, R0.10 - moderately resistant and R0.12 – susceptible. The four regions are 2 mm apart - region 1 indicates distal tissue, and region 4 was the furthest from the root tip. Bars are means of six replicates \pm SE ($\alpha=0.05$). Same letters indicate no significant difference.

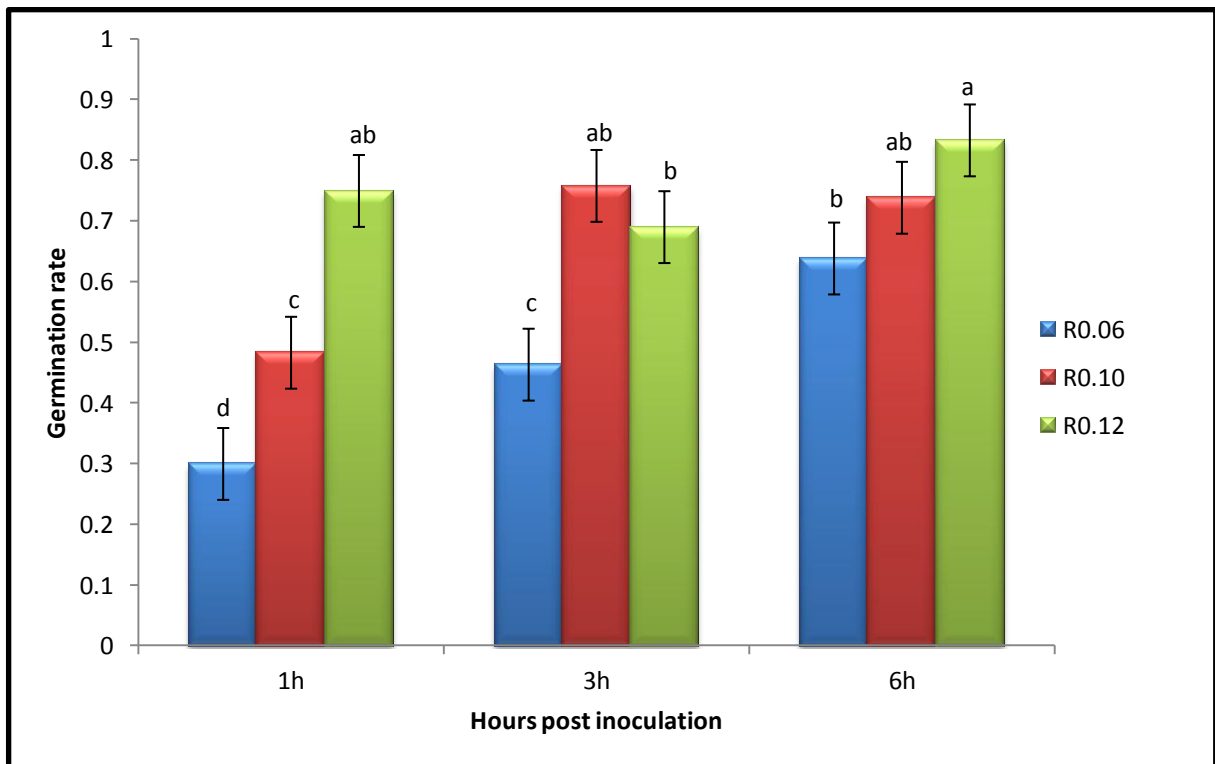


Figure 2.7. *Phytophthora cinnamomi* zoospore cyst germination rates on three avocado rootstocks varying in resistance (R0.06 – resistant, R0.10 – moderately resistant and R0.12 – susceptible). Bars are means of four replicates \pm SE ($\alpha=0.05$). Same letters indicate no significant difference.

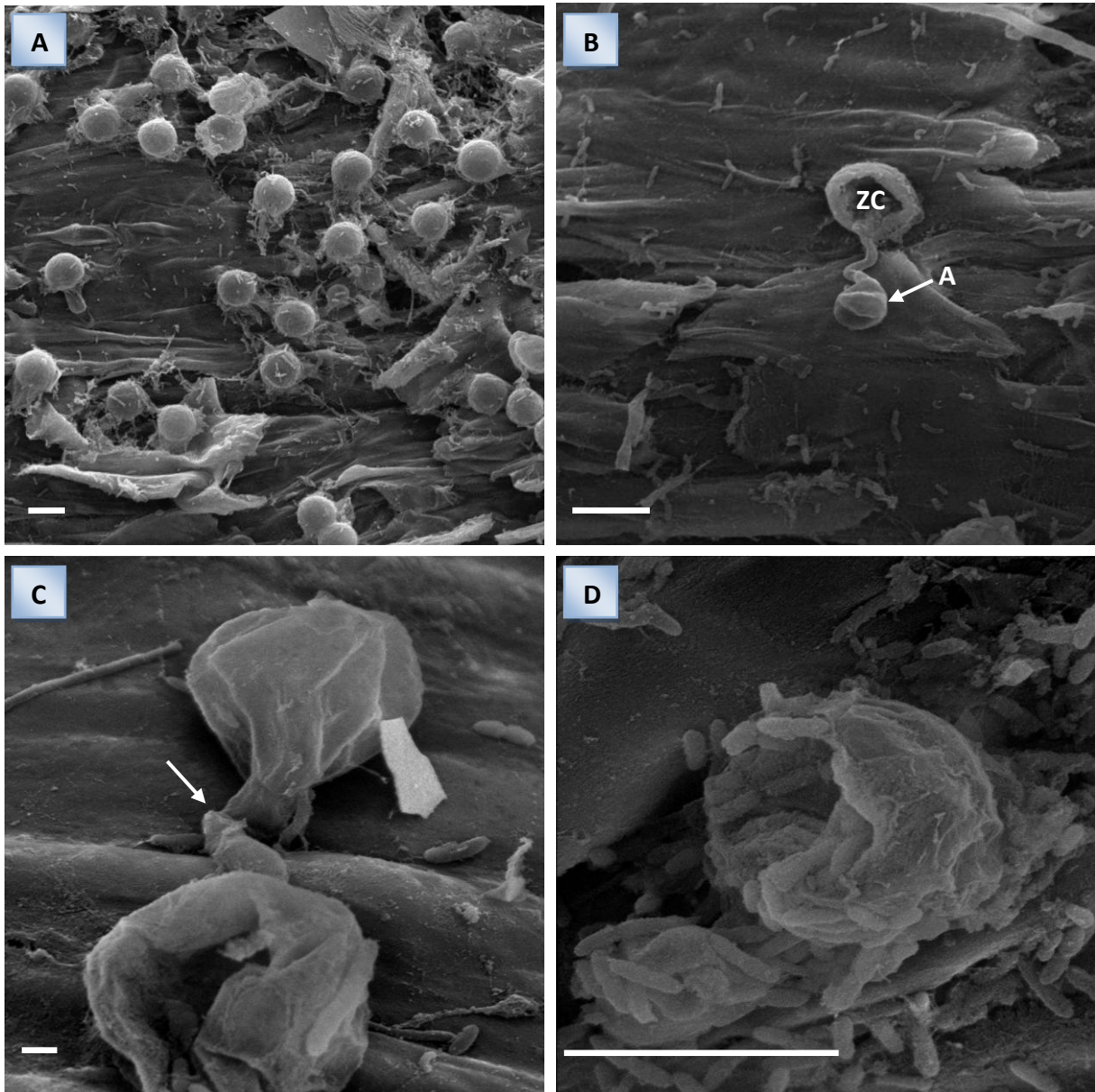


Figure 2.8. Scanning electron micrographs of *Phytophthora cinnamomi* zoospores encysted on avocado roots 1 hour post inoculation. **A.** Cysts (mostly ungerminated) on an R0.06 root. Scale bar = 10 μ m. **B.** Germinated cyst forming an appressorium (A) on an R0.06 root. Scale bar = 10 μ m. **C.** Two germinated cysts, with hyphae entering an R0.10 root at the same site via direct penetration (arrow). Scale bar = 1 μ m. **D.** Bacteria present in abundance around a degraded encysted zoospore. Scale bar = 10 μ m.

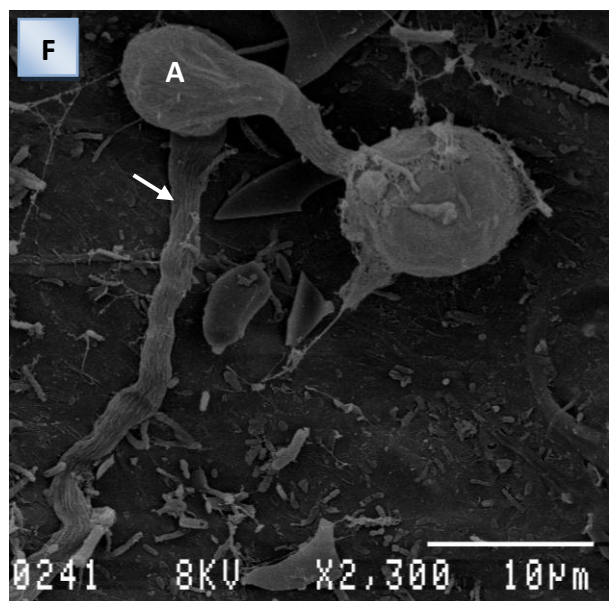
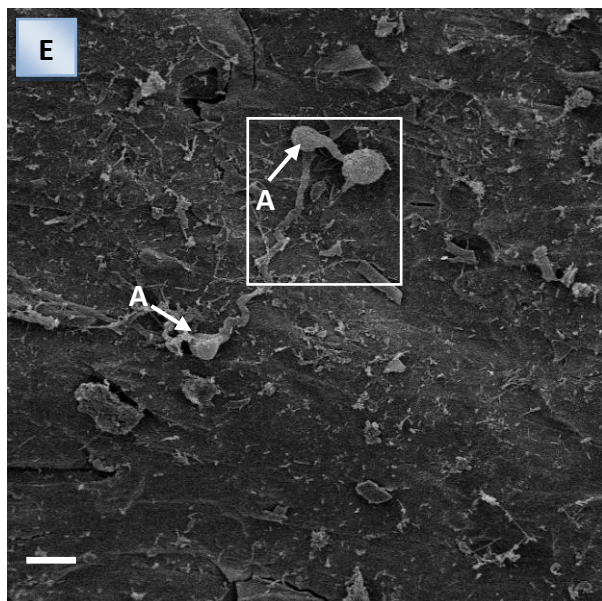
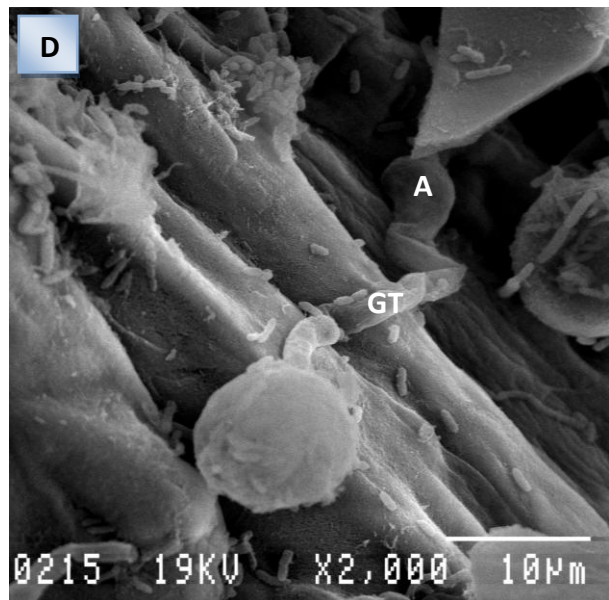
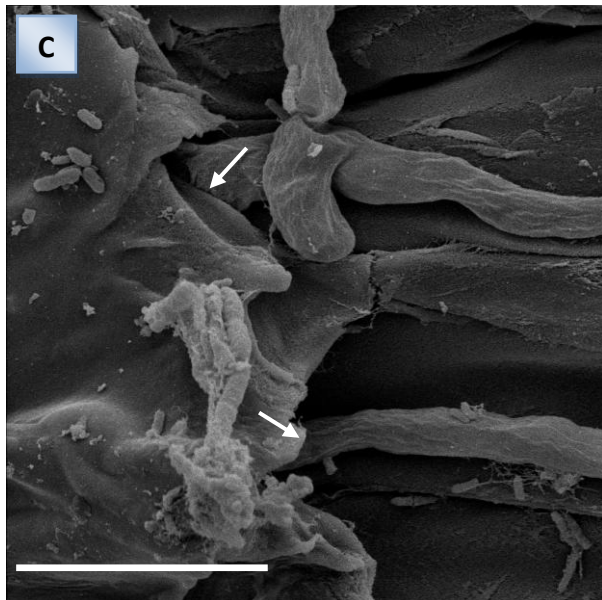
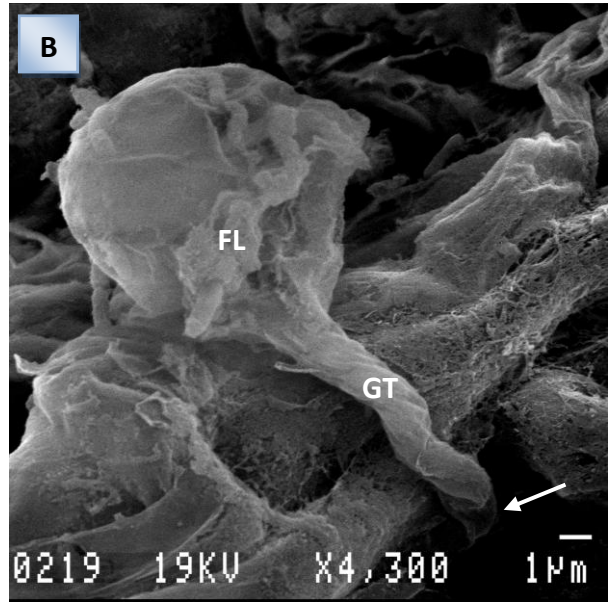
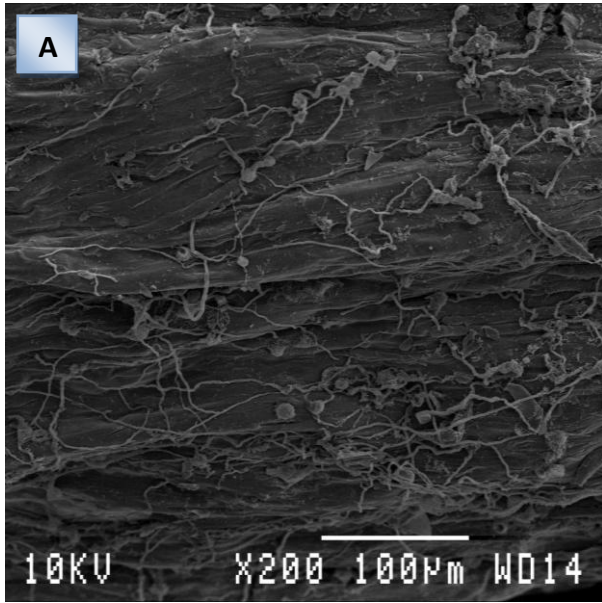


Figure 2.9. Scanning electron micrographs of *Phytophthora cinnamomi* zoospore cysts on avocado roots 3 (**B & D**) and 6 (**A, C, E & F**) hours post inoculation. **A.** R0.10 root overgrown by *P. cinnamomi* hyphae. **B.** Germinated cyst with a flagellum (FL) still attached on an R0.10 root, with a germ tube (GT) growing indirectly into an opening (arrow). **C.** *Phytophthora cinnamomi* hyphae growing into openings (arrows) in the root epidermis. Scale bar = 10 μ m. **D.** Germinated cyst on an R0.06 root containing a long germ tube and a terminal appressorium. **E.** Germinated cyst with intercalary appressorium formation, but unsuccessful penetration into the epidermis on an R0.06 root. A secondary hypha emerged from the appressorium which in turn produced a terminal appressorium. Scale bar = 10 μ m. **F.** Inset of **E.** The arrow indicates the secondary hyphae.

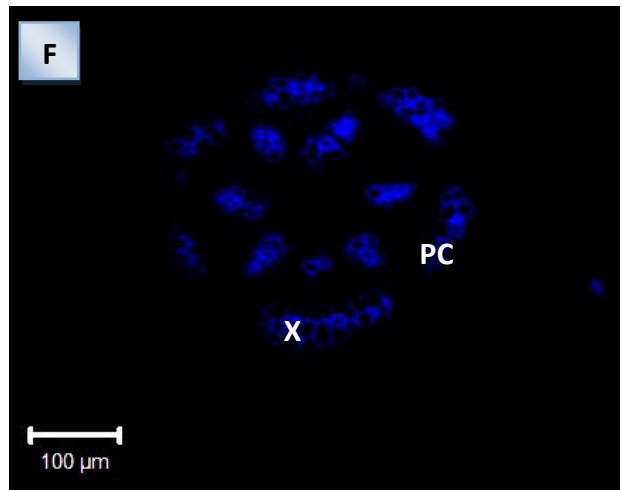
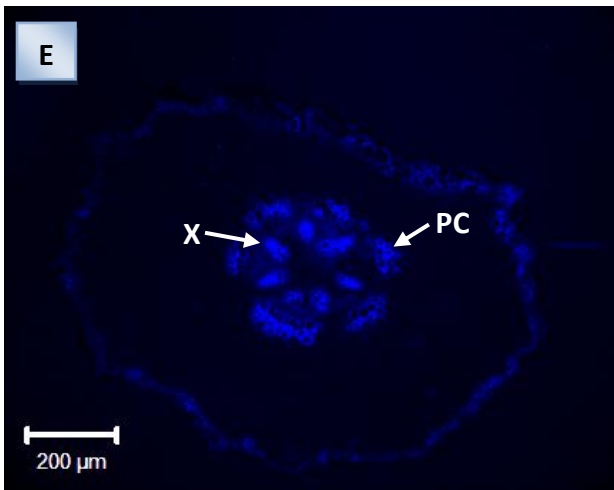
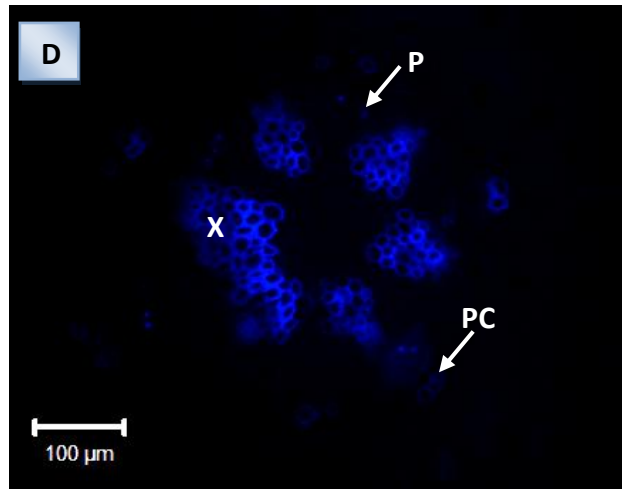
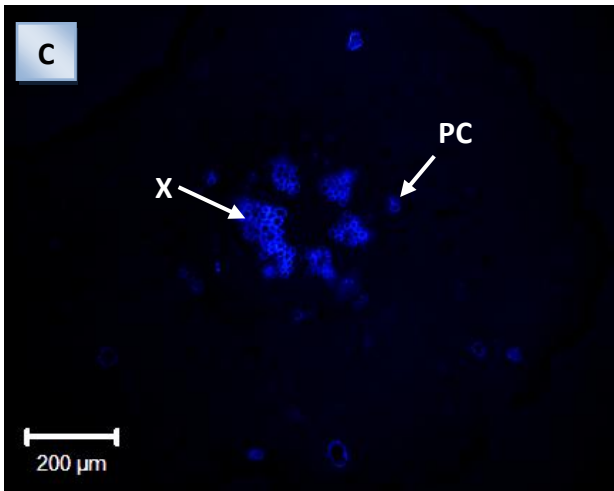
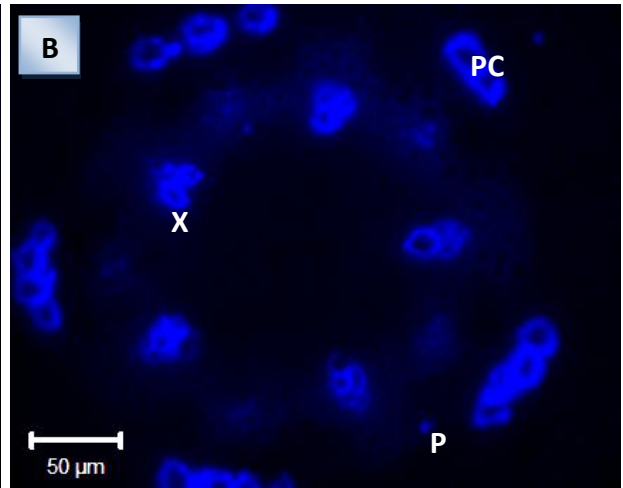
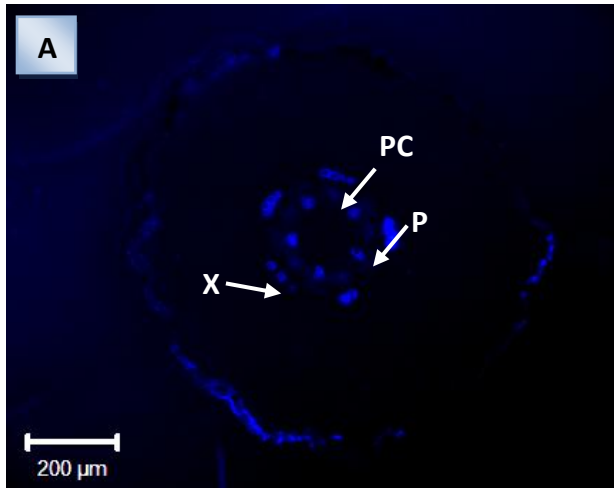


Figure 2.10. Fluorescent light micrographs of transverse sections of roots of three avocado rootstocks varying in resistance to *Phytophthora cinnamomi*, 10 mm from the root tip, stained with calcofluor. **A & B.** Resistant rootstock, R0.06. Vascular bundles [Xylem (X) and phloem (P)] and pericycle cells (PC) are indicated. **C & D.** Moderately resistant R0.10 root with abundant xylem vessels, but few phloem bundles. **E & F.** Susceptible R0.12 root. Xylem vessels are well-defined, but phloem bundles are minimal.

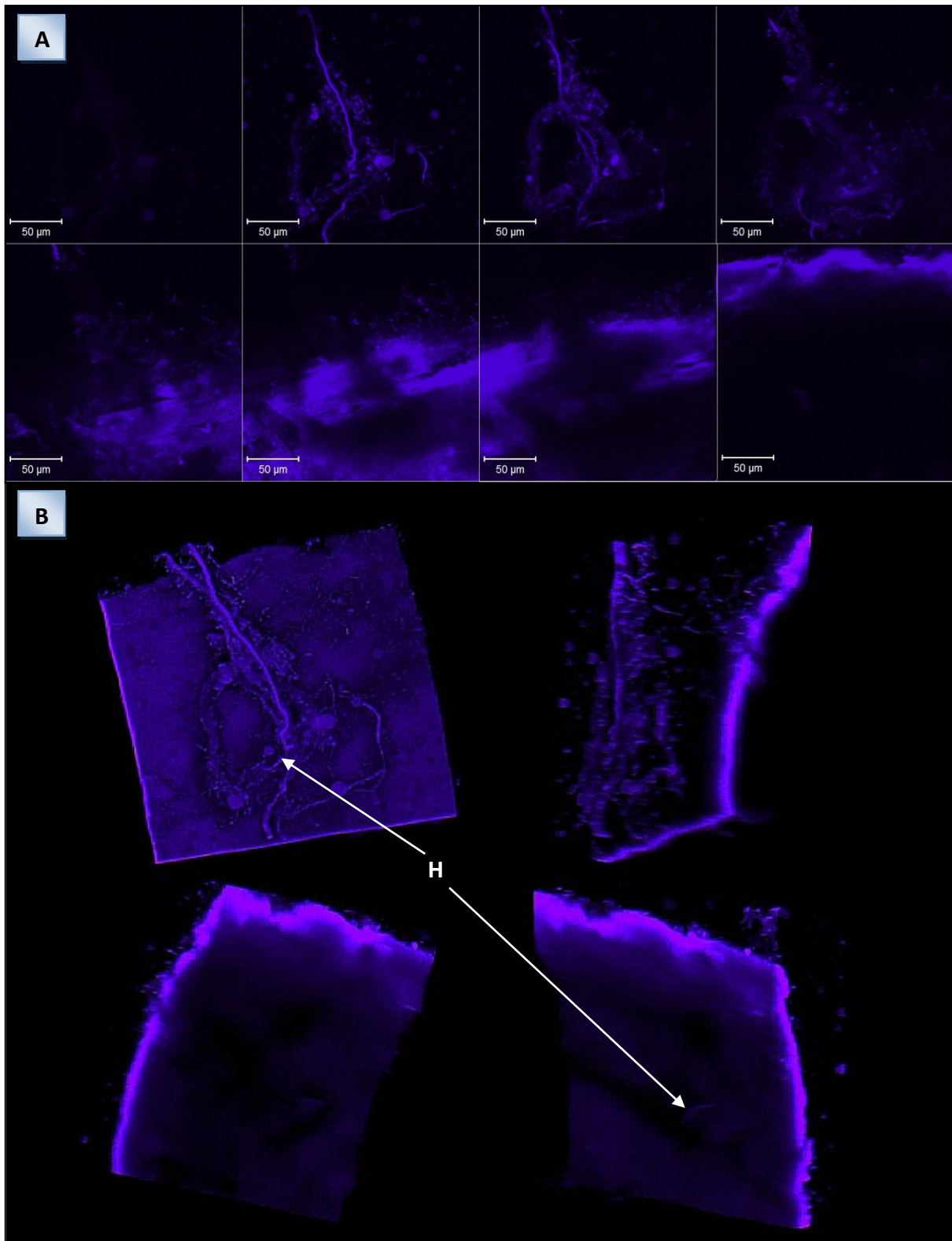


Figure 2.11. **A.** Subset of a z-stack (every 10th image) obtained by confocal imaging of an R0.10 avocado root 6 hours post inoculation with *Phytophthora cinnamomi*, stained with calcofluor. **B.** Three-dimensional projection of the entire z-stack shown in **A**, obtained by confocal imaging. Note the two arrows which point to *Phytophthora cinnamomi* hyphae (H) which penetrated the epidermis, on exactly the same, rotated region on the root.

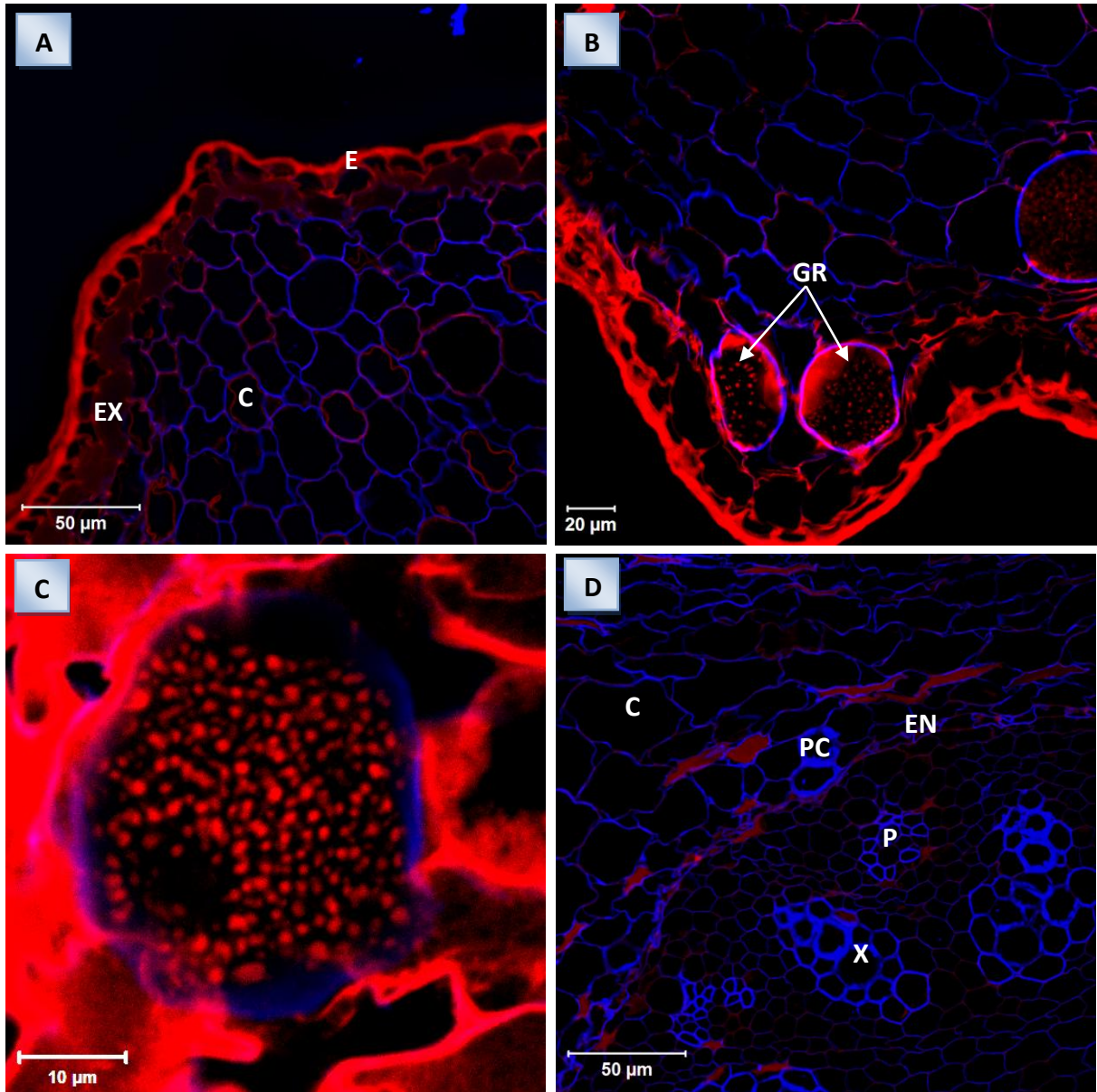


Figure 2.12. Confocal images of transverse sections of control, uninoculated avocado roots. **A.** R0.12 root. The epidermis (E), the exodermis (EX) and the cortex (C) are indicated. **B.** Autofluorescence of granular structures (GR) in a R0.12 root. **C.** A typical cell which contains fine granules (as in **B**), often present in avocado roots. **D.** Vascular bundles (Xylem, X and Phloem, P) and cortex of an R0.10 root. The endodermis (EN) is clearly defined in this micrograph. Pericycle cells (PC) are present in clusters in the endodermis.

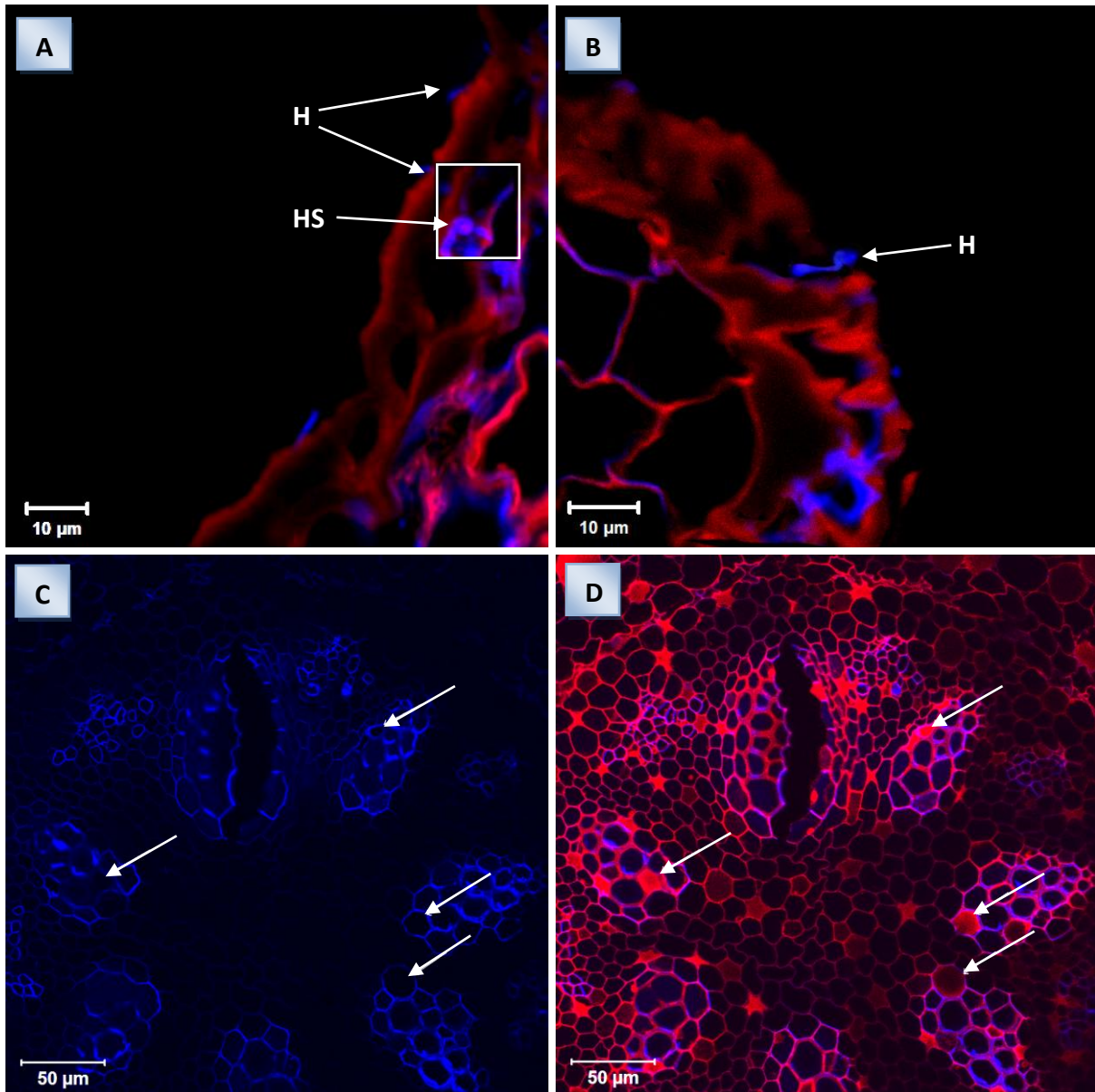


Figure 2.13. Transverse sections of avocado roots 3 hours post inoculation with *Phytophthora cinnamomi*, viewed by confocal microscopy. **A.** Penetrating hyphae (H) on the surface of an R0.12 root. The white rectangle is an inset from the same image at a focus point 7 μm deeper, revealing a haustorium-like structure (HS). **B.** Hyphae penetrating through the epidermis of an R0.10 root. Note the absence of cell walls directly adjacent to the hyphae. **C & D.** Tyloses of xylem vessels of an R0.10 root. **C.** Blue (405 nm) laser channel, indicating the absence of structures in selected cells, but overlaying of the red (543 nm) channel (**D**) revealed the presence of tyloses in these cells.

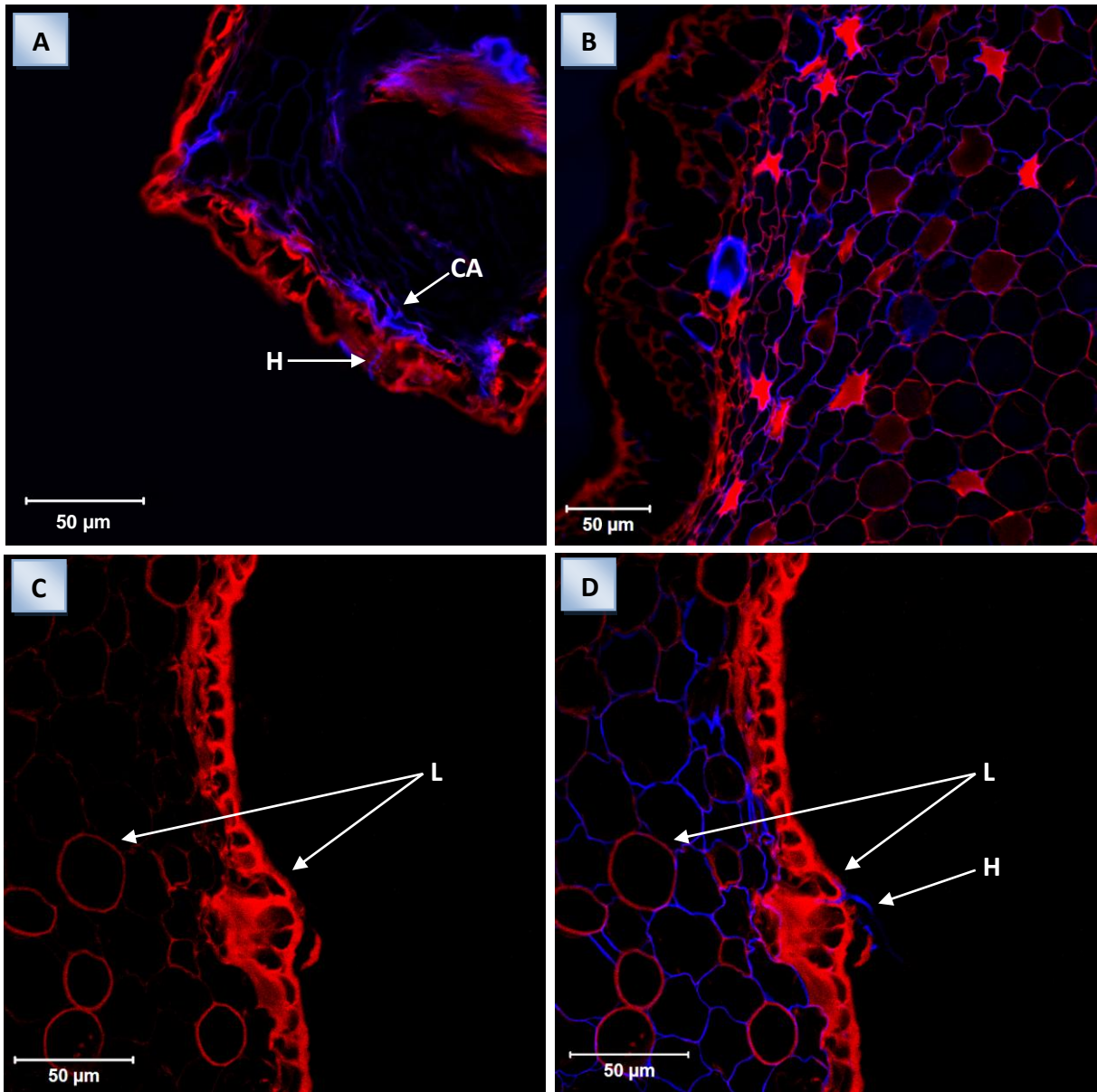


Figure 2.14. Confocal images of transverse sections of avocado roots 6 hours post inoculation with *Phytophthora cinnamomi*. **A.** Hyphae (H) of *P. cinnamomi* penetrating the epidermis of a resistant R0.06 root, with callose deposits alongside the adaxial exodermis near the site of penetration. **B.** R0.10 root showing a callose papilla near the epidermis (bright blue). **C.** Lignifications (L) of susceptible R0.12 cortical and epidermal cells. **D.** Same as **C**; an overlay of the red and blue channel, revealing penetrating hyphae (H) at the lignified area in the epidermis of an R0.12 root.

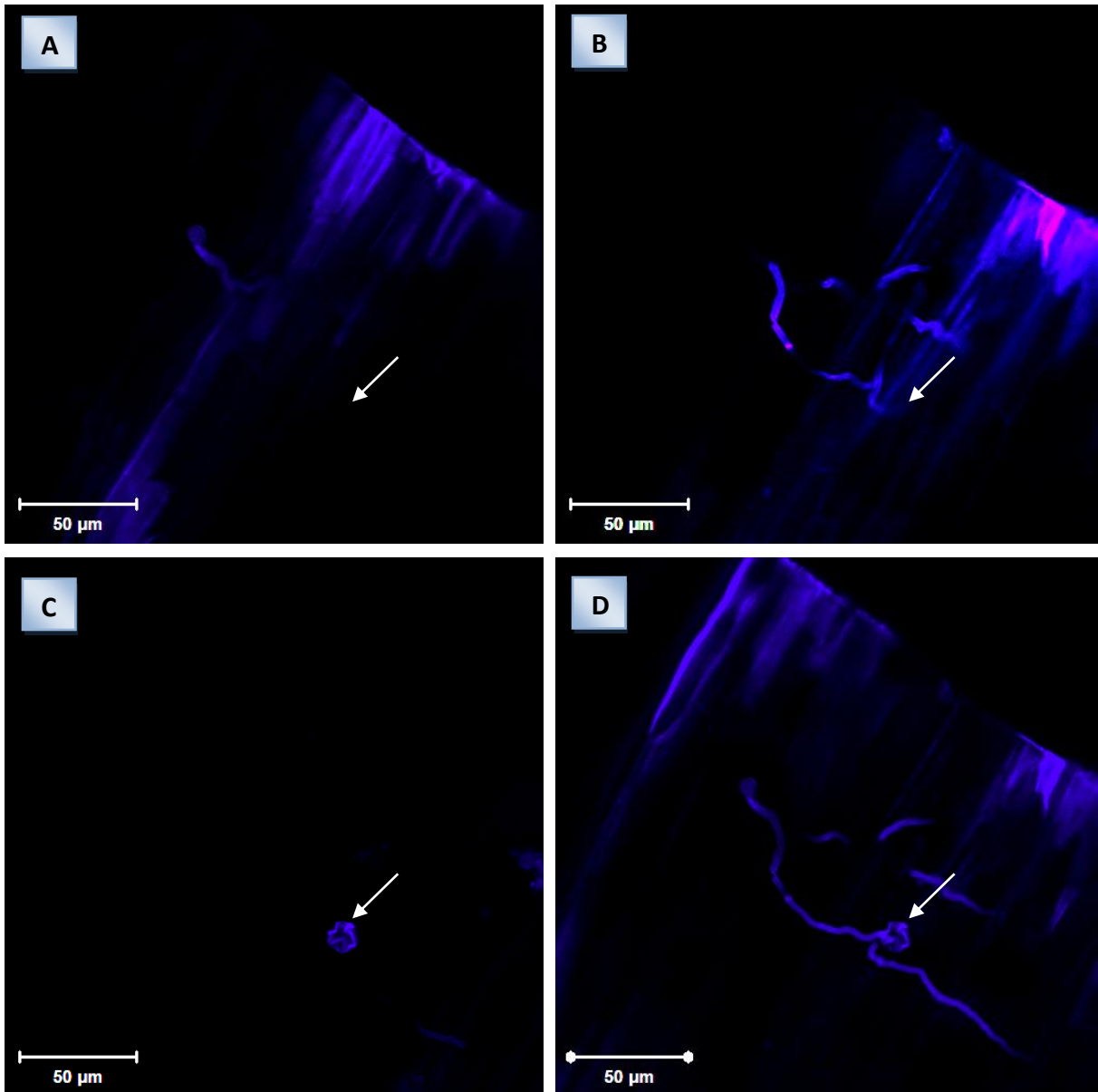


Figure 2.15. Z-stack of confocal microscopy images of a resistant R0.06 avocado root surface 6 hours after infection with *Phytophthora cinnamomi*. **A & B.** Hyphae of *P. cinnamomi*. **C.** Fissure or hole in epidermal cells, pointed out by an arrow. **D.** Combined images of the z-stack, indicating *P. cinnamomi* hyphae emerging through the degraded epidermis.

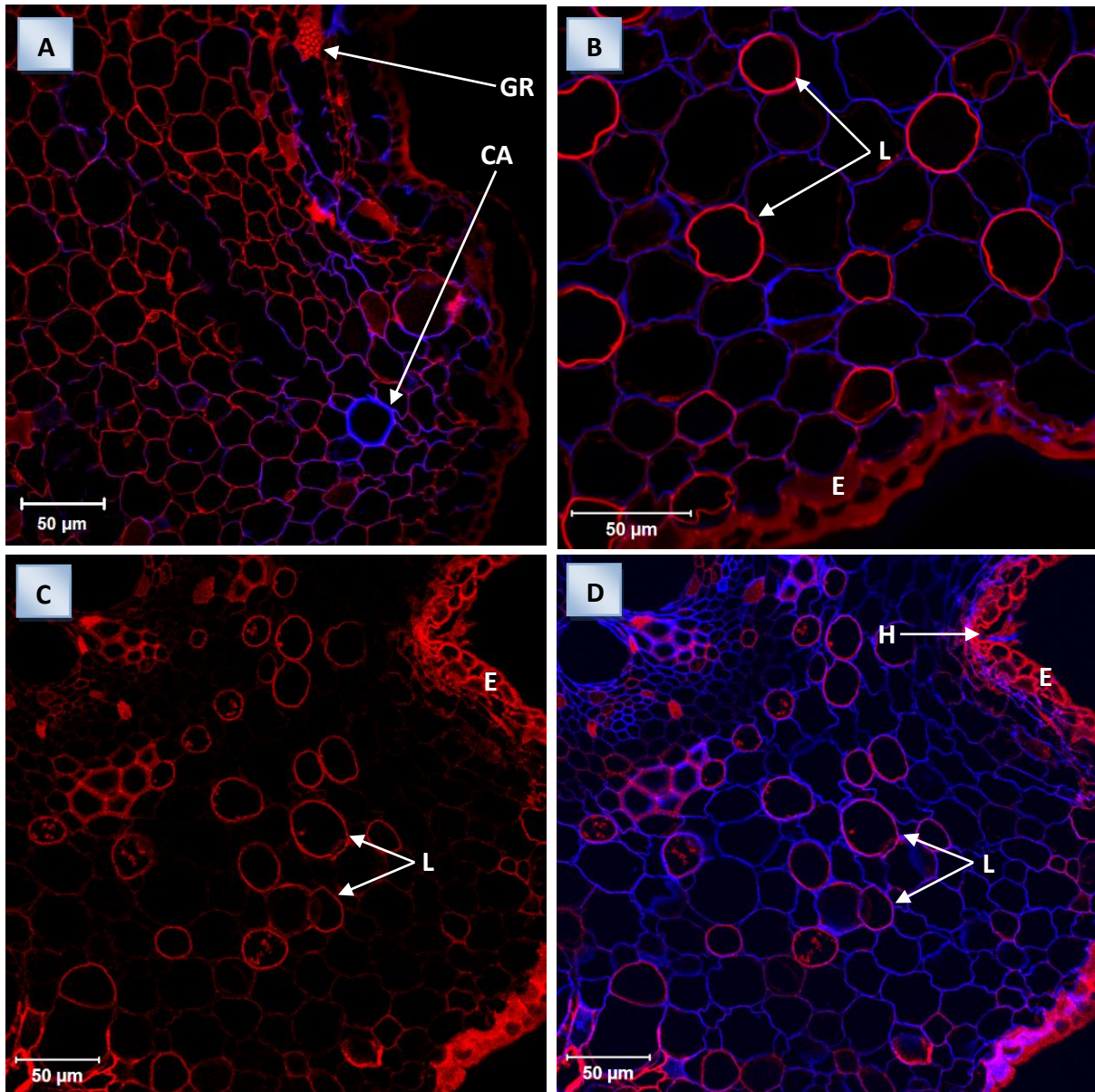


Figure 2.16. Confocal images of transverse sections of avocado roots 12 hours post inoculation with *Phytophthora cinnamomi*. **A.** Granules (GR) and callose (CA) in the cell wall of a resistant R0.06 root cortex. **B.** Lignifications in a susceptible R0.12 root cortex. **C.** Autofluorescent image of lignified cells (L) in an R0.10 root cortex. **D.** Same as **C**; an overlay image of red and blue channels, revealing hyphae (H) penetrating the epidermis (E).

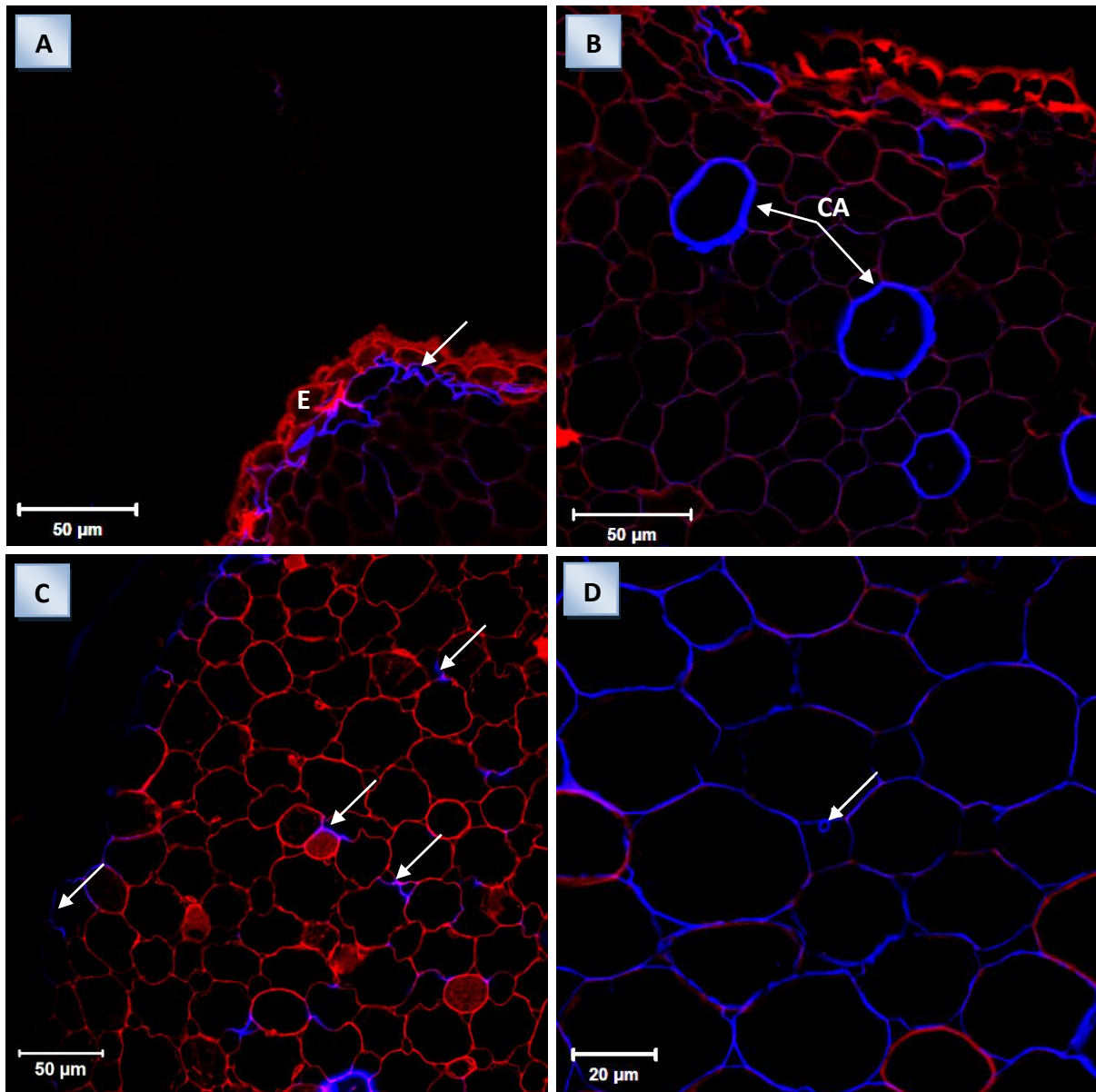


Figure 2.17. Confocal images of transverse sections of avocado roots 24 hours post inoculation with *Phytophthora cinnamomi*. **A.** Callose deposits (arrow) directly adjacent to the epidermis (E) of a resistant R0.06 root. **B.** Callose (CA) in R0.06 root cortical cells. **C.** Random areas in R0.10 cortex tissue fluorescing bright blue (arrows), as well as in cells adjacent to the epidermis. **D.** Intracellular hypha (arrow) in a susceptible R0.12 root cortex.

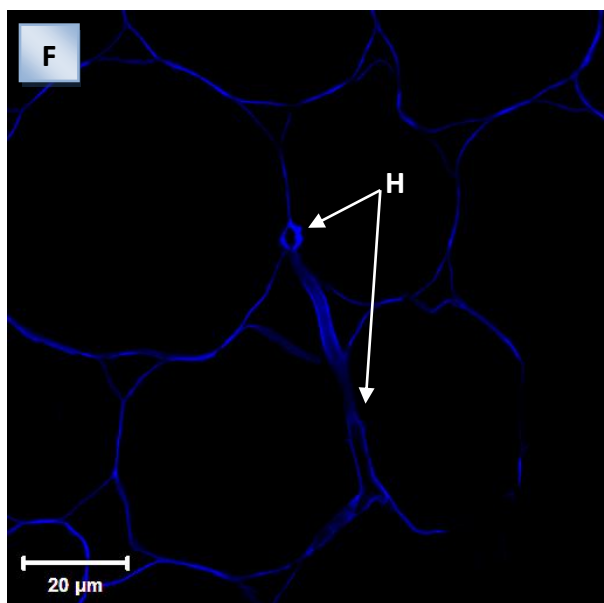
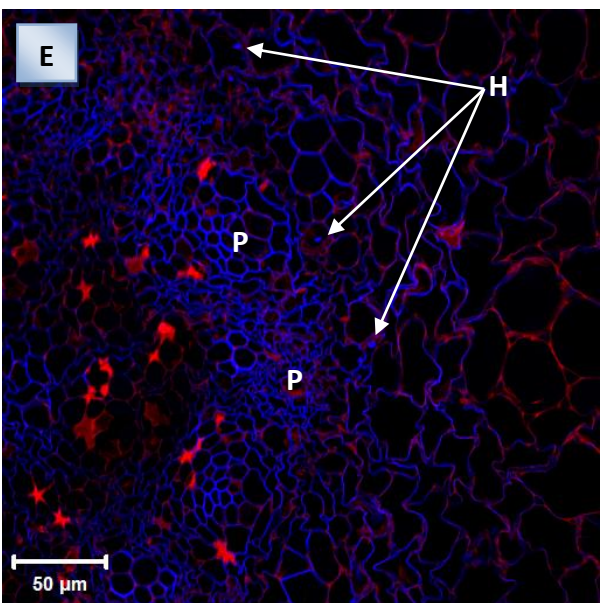
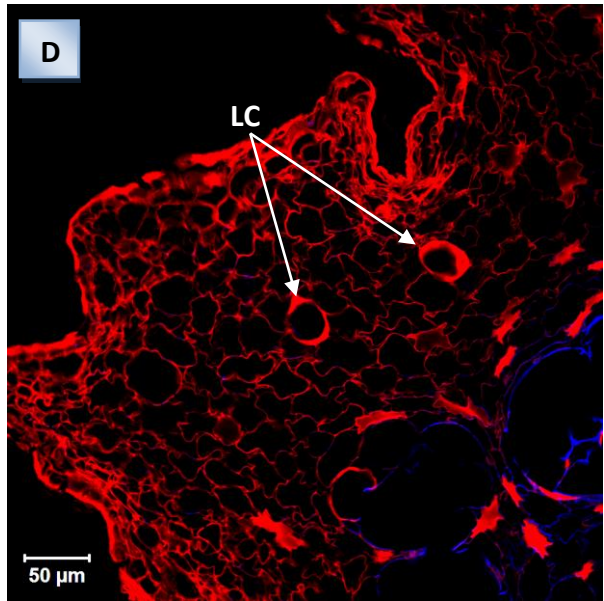
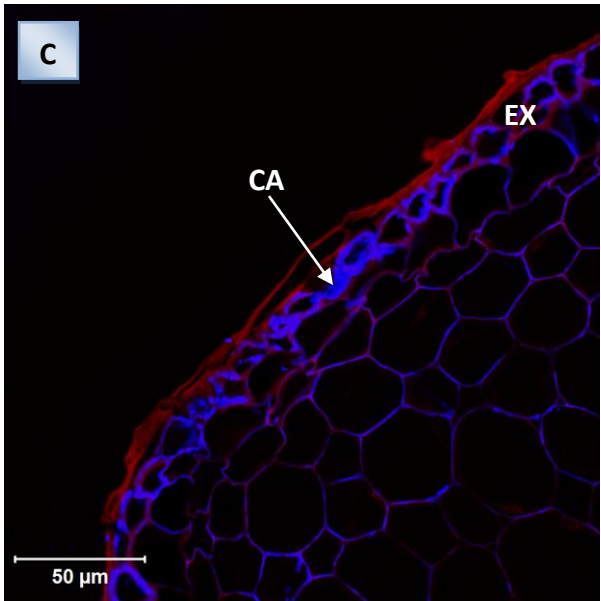
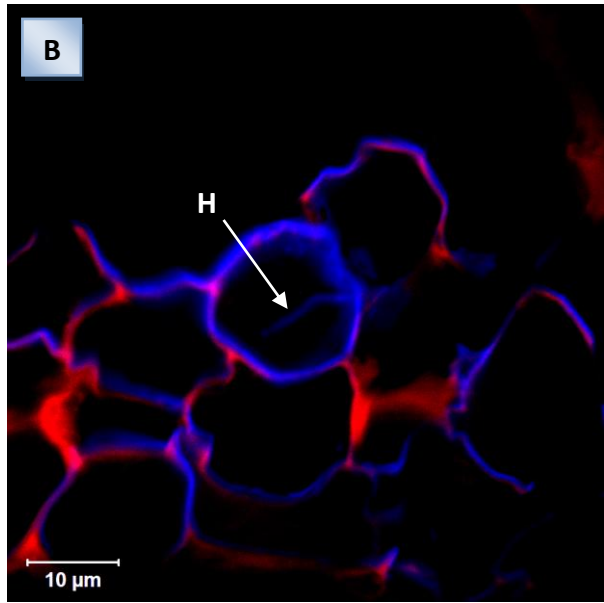
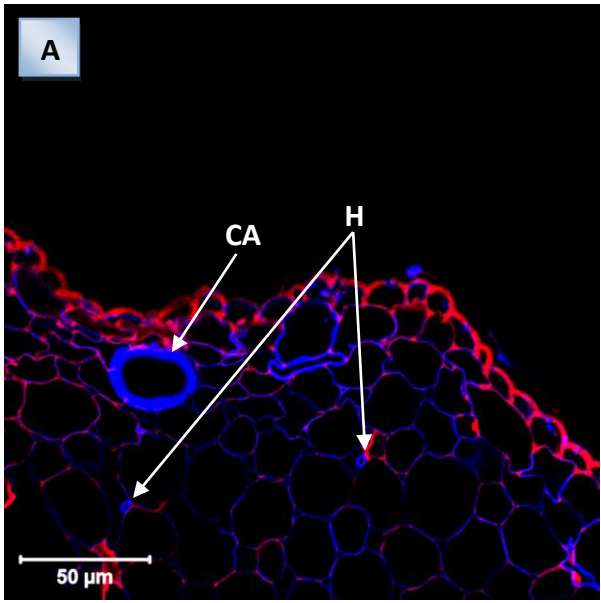


Figure 2.18. Confocal images of transverse sections of avocado roots 48 hours post inoculation with *Phytophthora cinnamomi*. **A.** *P. cinnamomi* hyphae (H) in cortex cells of a resistant R0.06 root. Callose (CA) thickening of cell walls is also visible. **B.** Infected parenchyma cell of an R0.06 root, displaying callose deposits around *P. cinnamomi* hyphae. **C.** An R0.10 root with callose deposits alongside the exodermis (EX). **D.** Lignified parenchyma cells in a susceptible R0.12 root cortex. **E.** Intracellular hyphae (H) near phloem bundles in an R0.12 root. **F.** Intercellular *P. cinnamomi* hyphae in a susceptible R0.12 root.

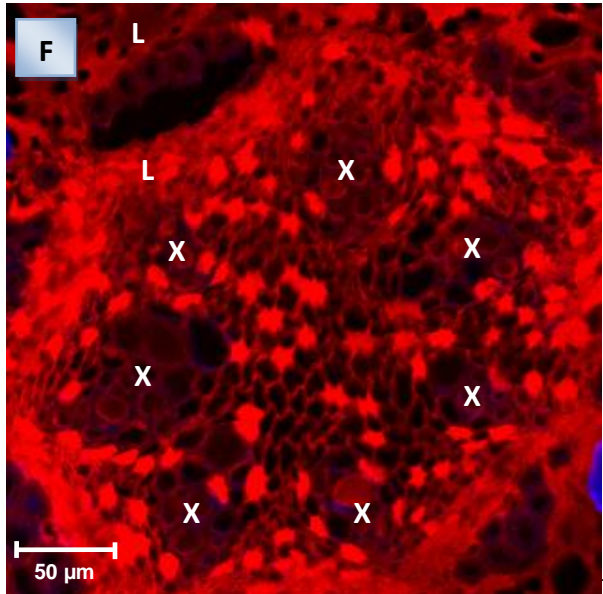
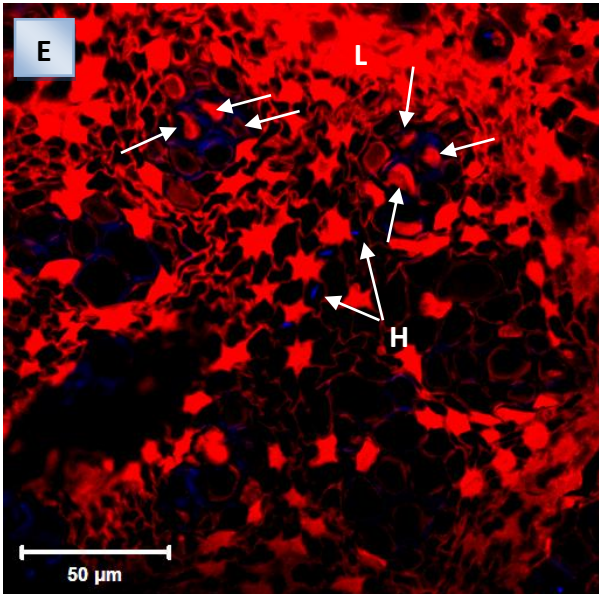
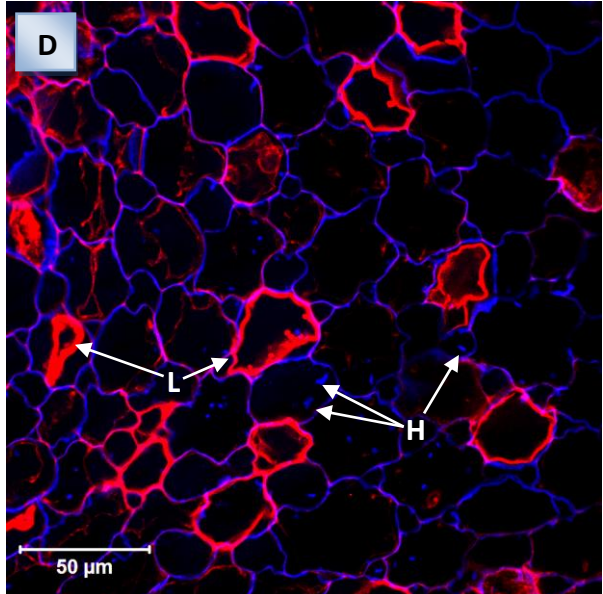
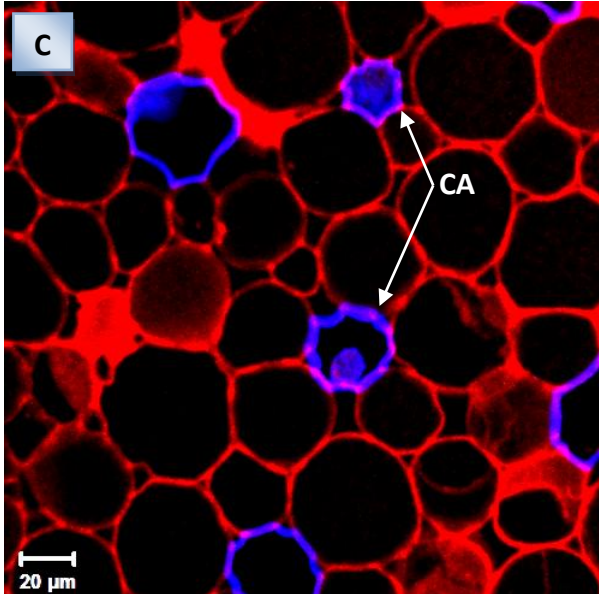
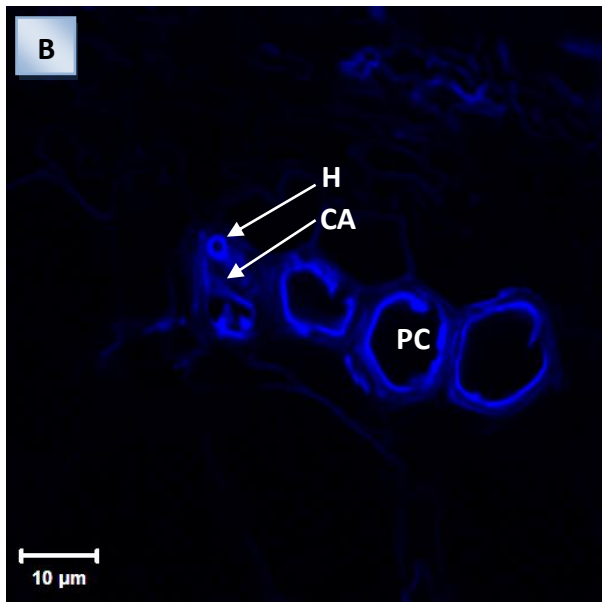
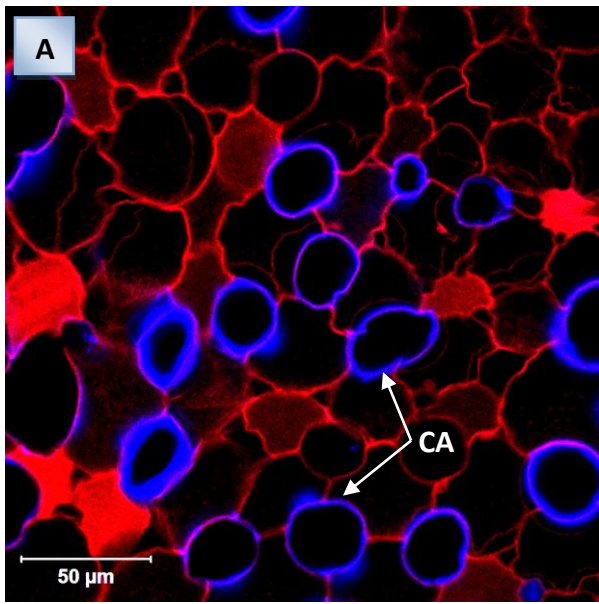
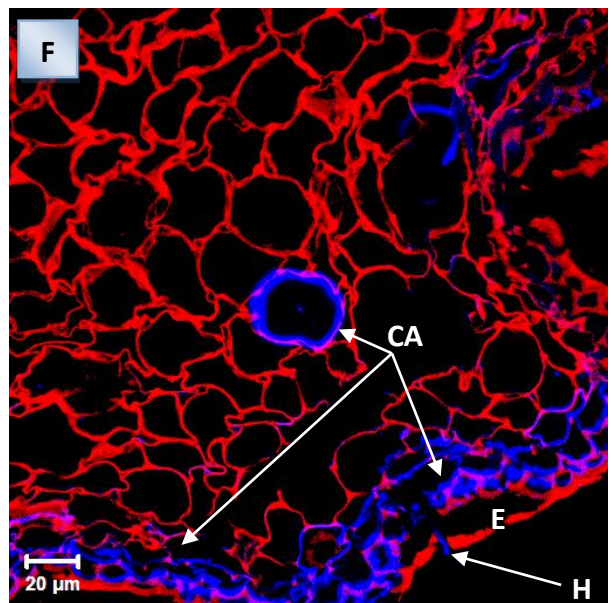
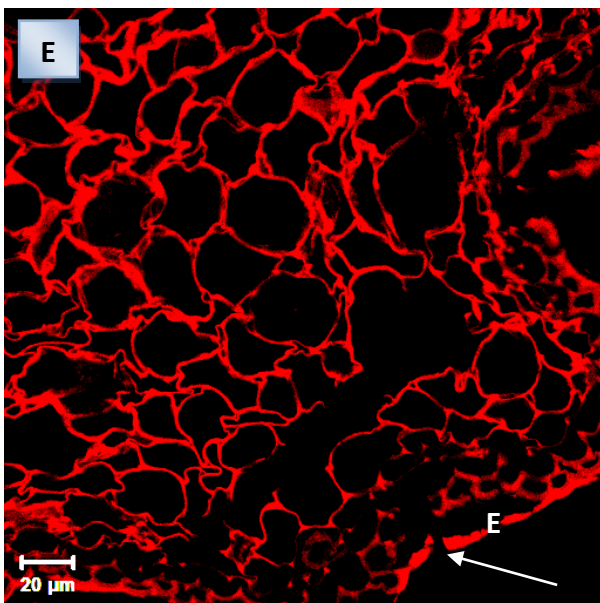
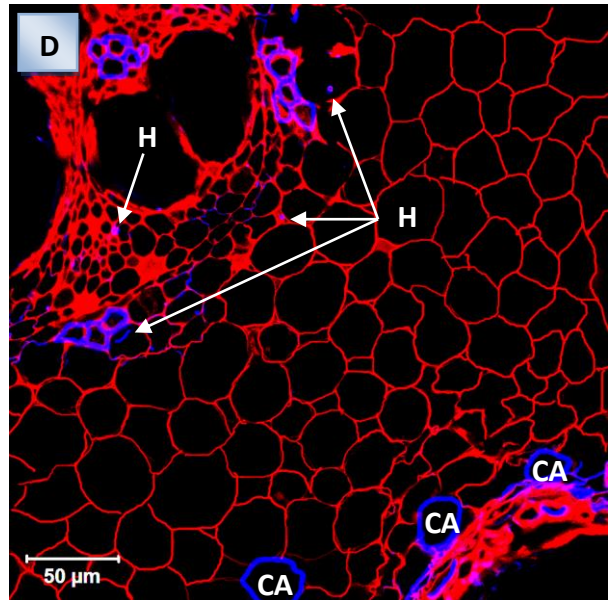
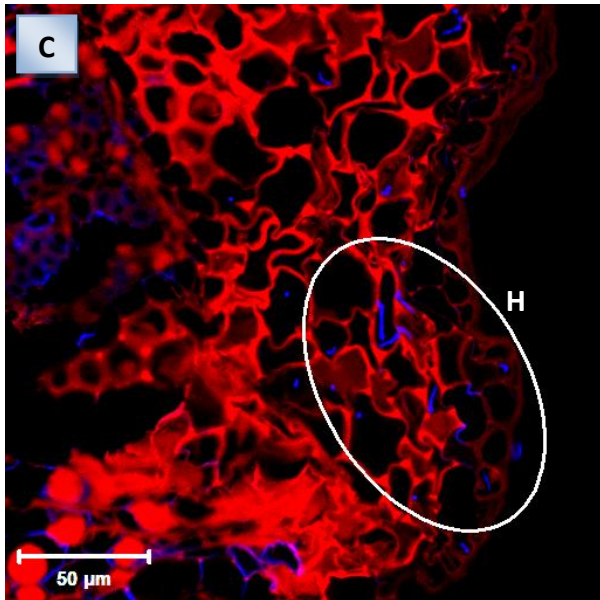
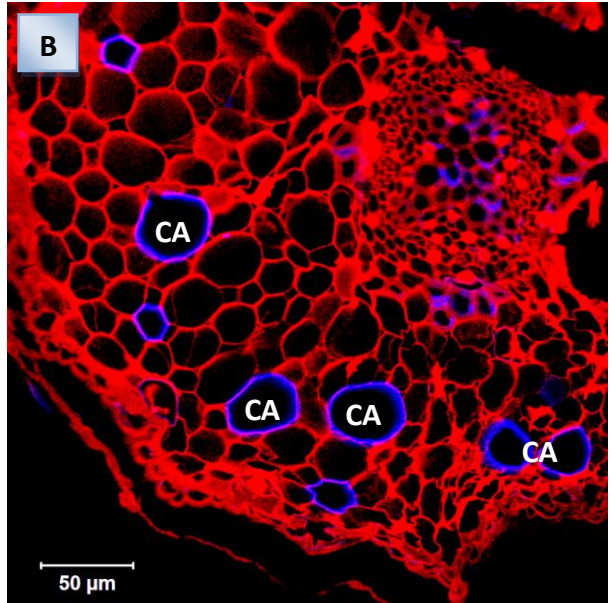
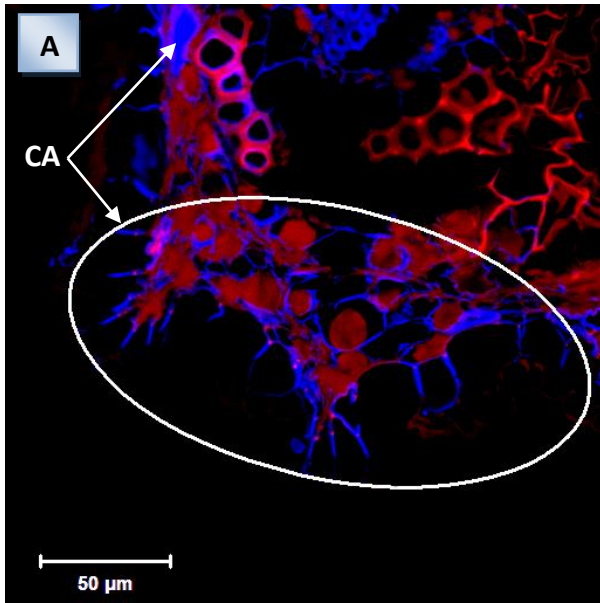


Figure 2.19. Confocal images of transverse sections of avocado roots 96 hours post inoculation with *Phytophthora cinnamomi*. **A & B.** Histological responses in resistant R0.06 roots. **A.** Callose-thickened walls of cortical cells (CA). **B.** Pericycle cell (PC) infected with *P. cinnamomi*. Callose papillae (CA) have formed around the hyphae (H). **C.** R0.10 root cortex cells with thickened cell walls. **D, E & F.** Susceptible R0.12 roots. **D.** Lignifications (L) of root cortex cells. Intracellular hyphae (H) are visible, growing predominantly in a lengthwise direction towards proximal tissue. **E.** Tyloses in xylem vessels (arrows), with lignified axial parenchymal cells surrounding vascular bundles. **F.** Xylem vessels (X) completely occluded by tyloses.



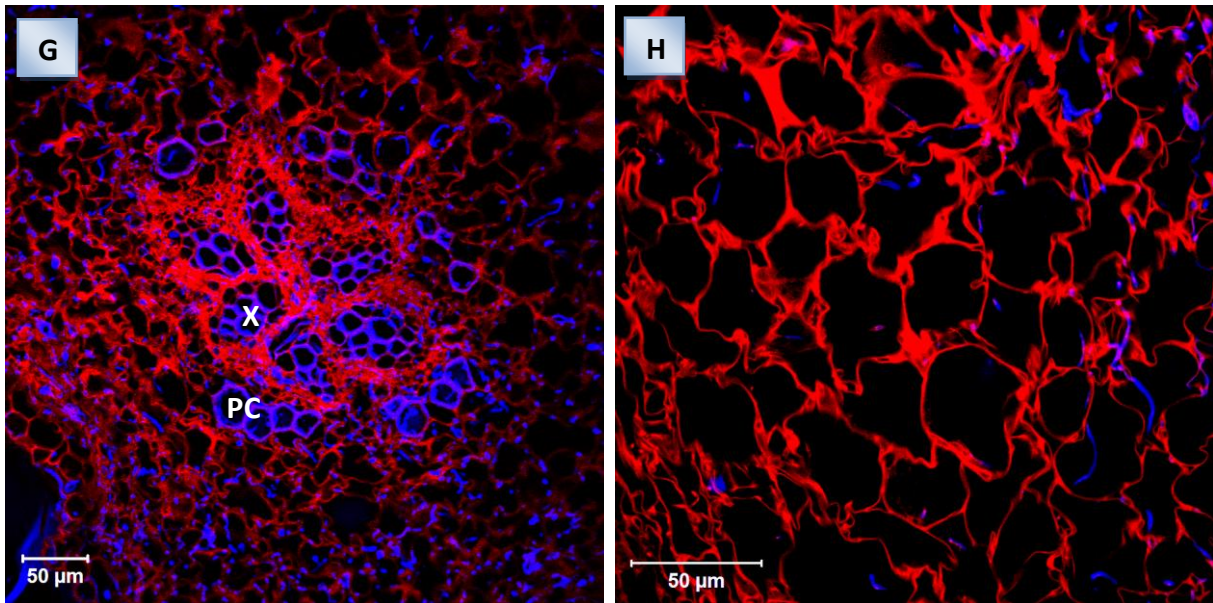


Figure 2.20. Confocal images of transverse sections of avocado roots 12 days post inoculation with *Phytophthora cinnamomi*. **A.** Callose depositions (CA) in cortical cell walls in a resistant R0.06 root. **B.** Fluorescence of calcofluor-stained cortical cells of a resistant R0.06 root. Note the absence of hyphae. **C.** Different area in the same root as **A**, with hyphae [blue staining; hyphae (H)] in the cortex. Note the absence of callose in this area. **D.** R0.10 root with callose depositions in the abaxial cortex. Hyphae are visible in the adaxial cortex in close proximity to the endodermis. **E.** Autofluorescent image of an R0.10 root. Note the fissure (arrow) in the epidermis (E). **F.** Same as **E**, with a 405 nm wavelength laser overlaid. Blue wall thickenings are visible in the abaxial cortex. A penetrating *P. cinnamomi* hypha is visible in the fissure [also shown in (E)]. **G.** Blue fluorescence of *P. cinnamomi* hyphae growing profusely in a susceptible R0.12 root. Xylem (X) and pericycle cells (PC) are also visible by blue fluorescence. **H.** *Phytophthora cinnamomi* hyphae (blue) in an R0.12 root cortex. Note the absence of calcofluor-stained cells.

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Table 2.1. A comparison between average xylem vessel areas in three avocado rootstocks varying in resistance to *Phytophthora cinnamomi* (R0.06 – resistant, R0.10 – moderately resistant and R0.12 - susceptible). Same letters in a column indicate that samples are not significantly different according to Student's pairwise T-test ($\alpha = 0.05$).

Rootstock	R 0.06	R 0.10	R 0.12
Average xylem area (μm^2)	5028.784 a	7560.618 ab	12939.814 b

Chapter 3

**Biochemical responses of three avocado rootstocks after challenge by
*Phytophthora cinnamomi***

Abstract

Despite decades of research, Phytophthora root rot (PRR), caused by *Phytophthora cinnamomi*, is still the most serious disease in avocado orchards around the world. The development of completely resistant rootstocks is a major target for those who would improve production of this crop. The objectives of this study were to associate several defense-related biochemicals with resistance to PRR in three rootstocks, R0.06 (resistant), R0.10 (moderately resistant), and R0.12 (susceptible). Phenolic acid content was determined in rootstocks planted in a field and a mistbed trial. In a separate greenhouse trial, ROS-scavenging enzymes and β -1,3-glucanase activities were determined spectrophotometrically. Phenolic acid expression did not differ between susceptible and resistant rootstocks in the field, but in the inoculation trial decreased after infection in resistant rootstocks, as opposed to an increase in R0.12. Ascorbate and guaiacol peroxidases decreased in R0.12 and in R0.10, respectively, but did not vary in R0.06. In all rootstocks, a trend towards decreased catalase activity occurred for the first 12 hours post inoculation, but R0.06 had a significant increase after 24 hours. R0.06 also had an early β -1,3-glucanase response 6 and 12 hours after infection, whereas activity in R0.10 and R0.12 was reduced. Increase in superoxide dismutase activity in R0.06 was more rapid than the more susceptible rootstocks, as a significant decrease occurred at 12 hours post inoculation. Effective regulation of defense-related molecules in avocado roots appears to be time, rather than quantity, related. Thus, rapid increases in superoxide dismutase and glucanases, in conjunction with lower APX, GPX and phenolic levels might yield a higher degree of resistance to *P. cinnamomi* in avocado plants. Results obtained from this study may lead to more efficient rootstock breeding.

Introduction

The pathogen *Phytophthora cinnamomi* Rands causes Phytophthora root rot (PRR) of avocado (*Persea americana* Mill.), which is a major threat to the avocado industry worldwide. Despite decades of research no definite control for this devastating disease has been achieved, and only partial resistant or tolerant rootstocks such as Dusa® are currently commercially available. Since these two organisms (host and pathogen) are native to different parts of the world, co-adaptation is not likely to have occurred (Whiley *et al.*, 2002). Nevertheless, it is clear that certain avocado cultivars are more resistant to *P. cinnamomi* (Kremer-Köhne & Duvenhage, 2000; Menge *et al.*, 1999).

Disease resistance in plants results from a variety of mechanisms. Physical or structural barriers are often associated with defense against pathogen invasions. These include responses such as tyloses, thickening of cell walls, lignification, necrophylactic periderm and callose depositions (Cahill *et al.*, 1989; Phillips *et al.*, 1987; Rookes *et al.*, 2008). Biochemical defense responses are more complex systems which are controlled by several major pathways in plants, such as the ethylene, jasmonate and salicylic acid pathways (Clarke *et al.*, 2000). Although structural barriers have been implicated in PRR resistance in avocado, Phillips *et al.* (1987) suggested that biochemical defense plays a role in contributing to the resistance observed in 'Duke 7' rootstocks. 'Duke 7' has been a standard rootstock in the avocado industry for several decades, but is currently being replaced by the resistant 'Dusa®' in many growing regions around the world. For the purposes of this study, tolerance is defined as the ability of a host plant to reduce the effect of a pathogen on plant fitness, health and yield; whereas resistance is referred to as the ability of a plant to restrict pathogen colonisation by means of an active defense response that results in an increase in plant health (Agrios, 2005; Kover & Schaal, 2002; Manners, 1993).

Upon pathogen invasion and recognition, plants defend themselves in a complex cascade of reactions. Phenolic acids and glucanase enzymes have antimicrobial properties, play a direct role in defense, and have been implicated in disease resistance in many host-pathogen interactions. Plants also protect themselves against harmful reactive oxygen species (ROS)

by producing peroxidases, catalases and superoxide dismutase. These countering enzymes could either protect the plant, or on the other hand benefit the pathogen by protecting it from the same harmful chemicals. Programmed cell death (PCD) could in the same way also either benefit the plant or pathogen; therefore, a fine balance and accurate response is needed for effective defense.

Phenolic acids are a well-known class of defense-related molecules. Phenylalanine ammonia-lyase, a key enzyme involved early in the phenylpropanoid pathway, has been implicated in increased lignin and phenolic acid production, although this response might often be slower than other defense responses (Cahill & McComb, 1992). Phenolic compounds are often stored in specialised cells in plants (Beckman, 2000). They are involved in many normal processes of differentiation in plants, but also play a role in disease resistance, either directly by being toxic to pathogens, or indirectly by being involved in other secretion pathways (Beckman, 2000; Cahill & McComb, 1992; Candela *et al.*, 1995).

Quick recognition of pathogens by plants often leads to a rapid response, called the hypersensitive response (HR). Pathogen-associated molecular patterns (PAMPs; small molecules excreted by microorganisms which are recognised by host plants) usually trigger the HR (Attard *et al.*, 2008; Dickinson, 2003). Although the HR has not been demonstrated in roots, defense response in roots is associated with an oxidative burst, similar to the HR, where ROS such as hydrogen peroxide and superoxide are released. This often leads to a phenomenon similar to PCD in animals, which is in essence self-sacrifice (Dickinson, 2003). Plants need a mechanism to protect themselves from these harmful chemicals. Enzymes involved in these processes include superoxide dismutase (SOD; which scavenges superoxide molecules) as well as peroxidases and catalases (hydrogen peroxide scavengers).

Peroxidases have a variety of functions in plants, some direct and others indirect. One example is lignin biosynthesis, whereby cinnamyl alcohols are oxidised using hydrogen peroxide as the final electron acceptor (García-Pineda *et al.*, 2010). Concurrently the plant is protected against ROS when hydrogen peroxide is converted to water and oxygen molecules. It has been proposed that vacuolar peroxidases can scavenge peroxide once the

hydrogen peroxide molecules diffuse into the vacuoles of plant cells by using phenolic compounds as primary electron donors (Takahama & Oniki, 1997). Conversely, it has also been shown that peroxidases can catalyse the formation of hydrogen peroxide (Peng & Kuc, 1992), which in turn can damage cells or tissue during oxidative burst.

Catalases are also anti-oxidative by scavenging hydrogen peroxide molecules. However, this enzyme does not need a co-substrate as in the case of peroxidases. Catalases have one of the highest turn-over rates of all enzymes, as one catalase molecule can convert several million hydrogen peroxide molecules to water and oxygen per second (Goodsell, 2004). Catalases are often associated with defense against pathogens in plants. For instance, in mango cultivars which are more resistant to the malformation pathogen *Fusarium moniliforme* J. Sheld., (currently *F. mangiferae*) a higher catalase activity was detected than in susceptible cultivars (Singh, 2006). Increased catalase activity was also observed in tobacco roots after inoculation with the arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. and Trappe. However, the opposite was observed by Takahashi *et al.* (1997) in antisense catalase transgenic tobacco plants, where plants with the most severe catalase depression were resistant to *Tobacco mosaic virus* (TMV). These findings suggest that plants defend themselves differently against biotrophs, necrotrophs and hemibiotrophs, as in the case of *P. cinnamomi*.

Superoxide dismutase is also a ROS scavenging enzyme and protects living cells from oxidative damage caused by superoxide anions. Superoxide ions are important signal molecules, but can also cause direct damage to living cells including pathogens. Cytosolic SODs usually contain Cu and Zn in their active sites, where these metals serve as electron acceptors during the anti-oxidation process (Alscher *et al.*, 2002). It has been proposed that necrotrophic pathogens might benefit from ROS (Able, 2003; García-Pineda *et al.*, 2010) and therefore increased SOD would be beneficial to plants during oxidative burst when countering these types of pathogens.

Glucanases form part of a larger group of enzymes, namely cellulases. Various conformations of β -linked glucan chains are degraded by glucanases. β -1,3-glucanase is

known to be associated with defense against fungal pathogens since their cell walls consist of chitin as well as glucan chains. Oomycetes have cell walls that consist of mainly cellulose and other glucans, which are specifically β -1,3- and β -1,6- linked (Zevenhuizen & Bartnicki-Garcia, 1970). Mulching in avocado orchards is an essential PRR control practice. This system relies on natural soil inhabiting microorganisms that decompose cellulosic components of plant material. This, in turn, degrades the cell walls of oomycete organisms. Evidence exists that laminarase (β -1,3-glucanase) can significantly reduce infection of *P. cinnamomi* on avocado roots (Downer *et al.*, 2001).

There is limited information on the complex interaction between *P. cinnamomi* and avocado at the biochemical level. Although many defense-related enzymes have been studied in avocado plants, these studies mainly investigated aerial plant parts; almost no data are available for these enzymes in roots. For instance, previously avocado catalases were studied to determine their role in fruit quality associated with storage and ripening (Chace, 1922; Sharon-Raber & Kahn, 1983). Studies were also conducted to elucidate the role of SODs in avocado fruit (Baker, 1976; Beno-Moualem & Prusky, 2000), but no articles have been published regarding their occurrence in avocado roots. The presence of glucanases in avocado mulches, as well as cellulase activity in avocado fruit, have been documented (Downer *et al.*, 2001; Downer *et al.*, 2002; Tucker & Milligan, 1991), but no experiments have been performed to determine their roles in avocado roots *in vivo*. Although guaiacol peroxidase has been investigated recently in avocado roots (García-Pineda *et al.*, 2010), the exact role of this enzyme in PRR resistance is unknown, since susceptible seedlings were used and no comparison was made between rootstocks that varied in resistance. It is thus apparent that knowledge of the function of many defense-related compounds in avocado roots and their roles in defense against *P. cinnamomi* is currently unexploited territory; their investigation would improve understandings of PRR resistance in this host.

The objectives of this study were therefore to elucidate the role of known pathogen defense-related molecules in three avocado rootstocks that vary in PRR-resistance (R0.06, R0.10 and R0.12), namely phenolic compounds, peroxidases, catalase, superoxide dismutase and glucanase enzymes. We hypothesised that: *i*) phenolics would be produced in higher

quantities in resistant rootstocks than in susceptible rootstocks; *ii*) quantities of peroxidases and catalases would decrease during the initial stages of *P. cinnamomi* infection; *iii*) quantities of SOD enzymes would increase most rapidly in resistant rootstocks; and *iv*) higher quantities of glucanases would be produced in resistant rootstocks. Results obtained from this study will lead to a better understanding of biochemical defense mechanisms in avocado to infection by *P. cinnamomi*.

Materials and Methods

Production and maintenance of avocado plants

For mistbed and greenhouse experiments, avocado plantlets (rootstocks R0.06, R0.10 and R0.12) were clonally propagated and provided by Westfalia Technological Services in Tzaneen, Limpopo Province, South Africa. Once plants were approximately 8 months old, they were transported to greenhouses at the University of Pretoria. Plants were grown in black bags containing perlite growth medium (Chemserve Perlite (Pty) Ltd, Rieperpark, South Africa) and watered three times per week with a light mist spray. Individual plants were fertigated with 50 ml Hoagland's solution (Hoagland & Arnon, 1950) once every 2 weeks. Temperatures in the phytotron were $25^{\circ}\text{C} \pm 2$, relative humidity was approximately 40%, and diurnal 12/12h light was used.

Isolates

Phytophthora cinnamomi was isolated from diseased avocado roots in Tzaneen, using PARPH selective medium [10 mg pimaricin, 250 mg ampicillin, 10 mg rifampicin, 100 mg pentachloronitrobenzene (Sigma-Aldrich, Steinheim, Germany) and 50 mg hymexazol (Tachigaren, Sankyo Company, Tokyo, Japan) in 1 L water agar]. Cultures were maintained in 15 ml glass bottles containing sterile distilled water and lawn grass. *Phytophthora cinnamomi* isolates were positively identified by means of PCR using LPV3 primers (Reverse 5'-GTCCAAACCGACTCTTGCTCGATG-3' and Forward 5'-GTGCAGACTGTCGATGTG-3'; Kong *et al.*, 2003). Mating types were determined to be A2 following a modification of the method of Tooley *et al.* (1989), by plating out known *P. cinnamomi* mating type strains opposite unknown isolates at 20°C. Isolates used varied between experiments according to the ability

to produce zoospores.

Phenolic acid determination

Inoculum production, infection and harvesting

i) Field trial

In a field trial for resistance screening and yield evaluations, avocado rootstocks were planted in 1999 in an orchard highly infested with *P. cinnamomi*. The rootstocks that were investigated, R0.01, R0.09, R0.10 and R0.12, were all grafted with 'Hass'. Trees were approximately 8 years old at the time of root-harvest and planted on a slope facing South-East in a randomised block design. The design consisted of five blocks containing five experimental units per block. Two trees were selected randomly per block for sampling. Feeder roots were harvested and immediately placed on pelleted cardice in 50 ml Cellstar® conical tubes (Greiner Bio-One, Frickenhausen, Germany). Average disease ratings (on a rating scale of 0 – 10, where 0 = healthy and 10 = dead) were determined annually and kindly provided by Westfalia Technological Services.

ii) Mist bed trial

Due to availability of material, R0.09 could not be included in this study, and R0.06, also a resistant rootstock, was used for the remainder of the experiments. For the mist bed infection trial, three to four 5 x 5 mm blocks of *P. cinnamomi* grown on potato dextrose agar (PDA) were transferred to sterile 800 ml-flasks containing 8 g D-glucose and 0.8 g yeast extract and incubated on shakers at 24°C for 7 days at 130 rpm. Mycelial masses were half-dried on paper towel, 33 g was weighed and blended in 1 L distilled H₂O and diluted to a final volume of 65 L H₂O. The inoculum solution was mixed with 64 kg vermiculite (Hygrotech, South Africa). Four trees for each sampling time, namely 0, 3, 12, 24 and 96 hours post inoculation (hpi), per rootstock (R0.06, R0.10 and R0.12) were planted in the vermiculite containing the *P. cinnamomi* mycelia. All roots from 8 month-old plantlets were harvested. Roots were placed in 50 ml Cellstar® conical tubes, snap-frozen in liquid nitrogen and immediately placed on pelleted cardice (-78.5°C). Samples were lyophilised and stored

at 4°C until further experimentation. Representative plants of each cultivar were maintained and watered daily in the mistbed for 6 weeks to confirm disease development.

Phenolic acid assay

Phenolic acid concentrations were determined according to Bekker (2007) with slight modifications. Root samples were ground with an IKA® A11 basic grinder (IKA Werke, GMBH & Co., KG, D-79219 Staufen) to a fine powder. One milliliter of a cold methanol : acetone : water (7:7:1, v:v:v) solution was added to 0.05 g powdered plant sample, followed by a 5 min ultrasonification step, by means of an ultrasonic bath (VWR Scientific, West Chester, PA, USA). Samples were centrifuged for 1 min at 12 000 g. This procedure was performed a total of three times, and the supernatant was pooled each time and used as crude extract in the various assays. The solid material was retained for extraction of cell wall bound phenolic acids. For each phenolic assay, aliquots of 0.25 ml crude extract was used and treated accordingly. For non-conjugated phenolic acids, the crude extract was acidified by adding 25 µl HCl (1 M). For glycoside bound phenolic acids, 40 µl concentrated HCl was added before heating at 96°C for 1 h. Ester bound phenolics were extracted by adding 0.1 ml NaOH (2 M), left at room temperature for 3 h and hydrolysed by the addition of 40 µl HCl (1 M). For cell wall-bound phenolic acids, the dry mass of roots was gravimetrically determined, re-suspended in 1 ml NaOH (0.5 M) for 1 h at 96°C and the supernatant acidified to pH 2 by the addition of HCl. The supernatants were centrifuged for 5 min at 12 000 g. In each case, phenolic acids were extracted by the addition of 1 ml anhydrous diethyl ether. Samples were dried at 4°C and the precipitate re-suspended in 0.25 ml 50% aqueous methanol.

For quantification of phenolic concentrations, the Folin-Ciocalteu reagent (Bray & Thorpe, 1954) was used. A gallic acid standard curve was constructed using a dilution series of 10 – 1000 µg/ml methanol (Figure 3.1). Assays were performed in triplicate in 96 well ELISA plates (three technical replicates of each sample on the same plate; Nunc, Roskilde, Denmark). The reagent mixture was comprised of 170 µl dH₂O, 50 µl 20% Na₂CO₃, 25 µl Folin-Ciocalteu's phenol reagent (Merck, Darmstadt, Germany) and 5 µl crude plant extract. Plates were incubated at 40°C for 30 min and absorbances read at 690 nm using an ELISA

reader (Multiskan Ascent VI.24354 – 50973; version 1.3.1). Phenolic acid concentrations were expressed as gallic acid equivalents per gram dry root weight.

Determination of activity of defense-related enzymes: ascorbate peroxidase, guaiacol peroxidase, catalase, β -1,3-glucanase and superoxide dismutase

Inoculum production, infection and harvesting

A *Phytophthora cinnamomi* of the A2 mating type was cultured on 20% V8 agar (V8 juice supernatant was prepared by centrifuging it with 10 g/L CaCO₃ for 20 min at 2 000 g; 15 g agar/L). Zoospores were produced by placing three 5 x 5 mm blocks of inoculum in 90 mm Petri dishes before adding approximately 20-25 ml 2% V8 broth. Plates were incubated at 20°C for 4 days whereafter the mycelia were rinsed three times with distilled water. After rinsing, 20 ml non-sterile, double-filtered (using Whatman filter paper) stream water was added to each dish and incubated at 20°C under a fluorescent light at 350 nm for 24-48 hours. Zoospores were released approximately 1 hour after a 45 min cold shock at 4°C. Zoospore concentrations were determined with a haemocytometer after vigorous vortexing.

Three avocado rootstocks, R0.06 (resistant), R0.10 (moderately resistant) and R0.12 (susceptible), were inoculated by spraying 3 ml of a solution of $4 \times 10^4 \text{ ml}^{-1}$ zoospores of *P. cinnamomi* per plant with an Efekto® 550-ml spray can. After inoculation, plants were kept moist for 20 min with a frequent misty water spray in a large container lined with a plastic bag before planting into moist vermiculite. The roots of five plants per rootstock were harvested at 0 (control), 6, 12, 24, 48 and 72 hpi, snap-frozen in liquid nitrogen and stored at -80°C. Infection of *P. cinnamomi* was confirmed by staining fresh roots with 0.01% calcofluor white fluorescent brightener 28 (Sigma-Aldrich) and observing roots microscopically under UV light, using a Zeiss Axioskop microscope (Carl Zeiss Ltd., Munchen, Germany). Digital images were obtained by using a Zeiss Axiocam HRc digital camera and Axiovision 4.7 software.

Extraction of total proteins

All protein assays were performed using a Spectramax Plus 384 microplate reader and

Softmax Pro software (Version 5.2; Molecular Devices, Sunnyvale, California) in 96 well ELISA plates at room temperature, except where otherwise specified. Crude protein extracts were prepared and separate assays were performed using the same samples.

Root samples were ground with an IKA® A11 basic grinder to a fine powder. Proteins were extracted by adding 4.5 ml sodium phosphate buffer [10 mM, pH 7, containing 1 mM ascorbic acid (AsA; Sigma-Aldrich)] and 12.5 µl protease inhibitor (Protease Inhibitor Cocktail Set III; Merck) to 0.25 g freshly ground frozen root samples in 5-ml Beckmann Coulter centrifuge tubes (Beckman Coulter, Inc., Fullerton, CA). The reagent mixtures were mixed using a vortex for 2 min, ultracentrifuged for 2 h at 100 000 g, after which the supernatant was transferred to sealed tubes and stored at 4°C until further experimentation. The total protein content was determined using the method of Bradford (1976). Bovine serum albumin (BSA; Sigma-Aldrich) was used to construct a standard curve (Figure 3.2). The reaction mixture contained 160 µl crude extract (40 x diluted) and 40 µl Bio-Rad dye reagent (Bio-Rad, Hercules, CA). Absorbances were read at 595 nm.

Ascorbate peroxidase (APX)

Measurement and comparisons of APX was performed as described by Murshed *et al.* (2008). The reaction mixture was comprised of 185 µl 50 mM potassium phosphate buffer (pH 7.0, containing 0.25 mM AsA), 5 µl 200 mM H₂O₂ (Merck) and 10 µl crude protein extract. The reactions were performed in non-UV resistant ELISA plates and were initiated by the addition of H₂O₂ using a multichannel pipette and incubated for 5 min at 25°C. The experiment was conducted by monitoring the rate of ascorbate oxidation with a decrease in absorbance at 290 nm ($E = 2.8 \text{ mM cm}^{-1}$). For each rootstock, four plants were evaluated per sample time. The specific activity of APX was calculated and expressed as units mg⁻¹ protein.

Guaiacol peroxidase (GPX)

GPX activity was assayed according to a modification of the method described by García-Pineda *et al.* (2010; $E = 26.6 \text{ mM cm}^{-1}$). The reaction mixtures comprised of 198 µl guaiacol solution [0.25% guaiacol, (v/v; Sigma-Aldrich) in 10 mM sodium phosphate buffer (pH 6.0) and 0.125% H₂O₂ (v/v; Merck)] and 2 µl crude extract in 96 well microtiter plates (Nunc).

Reactions were measured for 2 min at 470 nm.

Catalase

Catalase activity was assayed according to the Worthington Biochemical Corporation protocol with slight modifications (<http://www.worthington-biochem.com/CTL/assay.html>, accessed 2011/01/31). UV-transparent ELISA plates were used with reaction mixtures containing 190 μl dH₂O, 5 μl plant extract and 5 μl 0.784 M H₂O₂. Reactions were monitored for a decrease in absorbance at 240 nm for 5 min as H₂O₂ was converted to H₂O and O₂ ($E = 43.6 \text{ mM cm}^{-1}$).

Superoxide dismutase (SOD)

The SOD assay from Ukeda *et al.* (1997) was adapted for a 96-well microplate (Nunc) by modifying respective reagent volumes. SOD specific activity was determined by assaying its ability to inhibit XTT-formazan production, with superoxide generated from a xanthine/xanthine-oxidase system. Each well contained 10 μl xanthine (Sigma), 5 μl EDTA (ethylenediaminetetraacetic acid), 13 μl xanthine oxidase, 8 μl XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) and 154 μl potassium phosphate buffer (pH 8; 50 mM; containing 0.3 mM sodium cyanide) and 10 μl crude extract, with water as blanks. Control reactions followed a linear increase in absorbance at 470 nm for approximately 8 min before a decrease in the absorbance slope was observed; crude samples were, therefore, calculated accordingly.

β -1,3-glucanase

An optimised micro-assay protocol was followed to determine glucanase activity, using laminarin (Sigma-Aldrich) as substrate (Ramada *et al.*, 2010). A glucose calibration curve was constructed using a 20 mM glucose solution, with dilutions ranging from 0 – 90% (Figure 3.3). Reactions were carried out in 96 well PCR plates (Axygen, Union City, USA) containing 10 μl crude extract, 20 μl of 0.25% laminarin. A sealing film (Axygen) was used to prevent evaporation. The mixture was incubated in a thermocycler (Eppendorf Mastercycler Gradient; Eppendorf, Hamburg, Germany) for 40 min before adding 100 μl DNS solution and incubation for 5 min at 95°C. The DNS solution contained 0.687% (w/v) dinitrosalicylic acid

(DNS; Sigma-Aldrich), 1.28% (v/v) phenol (Sigma-Aldrich), 19.92% (w/v) Na-K-tartrate (Merck) and 1.226% (w/v) NaOH (Merck). One unit was defined as the amount of enzyme needed to produce 1 μmol reducing sugar min^{-1} .

Statistical analysis

Data generated from the trials were subjected to an analysis of variance (ANOVA) using the General Linear Model Procedure from SAS software (Version 9.2) and Fisher's protected Pairwise Test (SAS Institute Inc. 2004).

Results

Phenolic acids

i) Field trial

Root rot symptoms observed in trees in the field included yellowing and wilting of leaves, stunted tree growth, sparse leaves on branches and in severe cases dead trees. Infected roots had typical black necrotic symptoms and were brittle. Since the first year of planting, disease ratings in the susceptible R0.12 rootstock were higher than the other three tested rootstocks (R0.01, R0.09 and R0.10). The R0.12 rootstock was rated 2.66 in 1999, and increased annually to 9.64 in 2008 when sampling for phenolic extractions was performed. Ratings for R0.01 and R0.09 averaged 2.31 and 2.13, respectively. R0.10 had an average of 3.61 and R0.12, the most susceptible rootstock, had an average disease rating of 8.92.

Detectable significant differences in phenolic acid content were observed in total soluble phenolic acids and cell wall bound phenolics. In both instances the R0.01 rootstock had lower phenolic levels than the other three rootstocks (R0.09, R0.10, R0.12; Table 3.1). No significant differences were found among the rootstocks in non-conjugated phenolic acids, ester bound phenolic acids and the glycoside bound phenolic acids. High variation in phenolic acid levels was obtained from the field trial, as responses (or lack of responses) could be influenced by environmental factors. However, statistical analysis indicated that the slope of the orchard did not influence the phenolic content.

ii) *Mist bed inoculation trial*

Six weeks after inoculation, disease symptoms were observed in all rootstocks. R0.06, the resistant rootstock, occasionally showed limited disease symptoms on roots. Severe symptoms were apparent on the more susceptible R0.12 roots (Figure 3.4). Single roots of R0.10 often showed more disease symptoms than those of R0.06, but not as severe as those of R0.12. Symptoms included dark brown to black roots as a result of necrosis and were frequently brittle.

Cell wall bound phenolic acids (CWBPA)

At 0 hpi (control plants) cell wall bound phenolics in R0.10 and R0.12 did not differ significantly from one another, but CWBPA were significantly lower in the resistant R0.06 (Figure 3.5). At 3 hpi, CWBPA started to increase in R0.12, and decreased in R0.10, with quantities in R0.06 remaining unchanged. The susceptible R0.12 had the highest CWBPA in response to *P. cinnamomi* infection at 12 hpi (mean value of 7.41 mg gallic acid equivalent per gram dry weight). In contrast, the resistant R0.06 had the lowest levels, although the moderately resistant R0.10 did not differ significantly from R0.06 at this time point. No significant changes in CWBPA levels occurred later than 24 hpi in R0.10. At 24 hpi, a slight increase took place in R0.06, but a significant decrease occurred at 96 hpi in both R0.06 and R0.12 although the latter did not differ significantly from the uninfected plants. At 96 hpi the mean CWBPA level in R0.06 was 1.59 mg gallic acid equivalent per gram dry weight, which was the lowest CWBPA value observed at all sample times and among all rootstocks.

Ester bound phenolic acids (EBPA)

Unique response curve trends for EBPA were observed between the two more resistant rootstocks R0.06 and R0.10, compared to the more susceptible R0.12 rootstock. The R0.06 rootstock showed little variation across all time intervals studied, and EBPA levels did not differ significantly from control plants at any point except at 24 hpi where EBPA were particularly low (Figure 3.6). No differences were detected between the three rootstocks at 0 hpi. At 3 hpi R0.10 showed a significant EBPA increase in response to *P. cinnamomi* and was higher when compared to the other two rootstocks as well as to other time points.

However, at 12, 24 and 96 hpi levels in R0.10 had decreased and were not significantly different from those observed in noninoculated control plants. R0.12 showed a change in ester bound phenolic amounts at 24 hpi when a significant increase took place followed by another significant increase at 96 hpi. The graph trend of the susceptible R0.12 rootstock was notably opposite to that of the resistant rootstocks.

Glycoside bound phenolic acids (GBPA)

There were no significant differences between R0.06 and R0.10 at 0 hpi, however, GBPA in R0.12 were approximately three-fold higher (Figure 3.7). A rapid decrease in quantities was observed in both R0.06 and R0.12 at 3 hpi, where R0.06 had a significantly lower value than R0.10 and R0.12. No significant changes were visible at 12 hpi compared to 3 hpi for each rootstock. GBPA levels in R0.06 remained low for the remainder of the trial. In R0.10 an increase in GBPA amounts occurred at 24 hpi with yet another increase at 96 hpi. In R0.12 a significant increase in GBPA production was only observed 96 hpi. Although GBPA in R0.06 was low in control plants, significant lower levels were detected after four days of inoculation, as opposed to GBPA levels which were particularly high in R0.10 and R0.12.

Non-conjugated phenolic acids (NCPA)

Constitutively, NCPA did not differ between resistant R0.06 and susceptible R0.12, but R0.10 had significantly higher non-conjugated phenolic levels (Figure 3.8). A rapid rise in NCPA production occurred in R0.06 at 3 hpi, where this rootstock had the highest NCPA levels at this time point. The opposite was observed in R0.10, as a decrease in phenolic levels took place; however, R0.12 still had the lowest NCPA levels. Significant decreases in NCPA amounts were observed for R0.06 at 12 and 24 hpi, which remained low until 96 hpi. NCPA levels in R0.10 also decreased at 12 hpi, but increased significantly at 24 hpi, which allowed phenolic levels in R0.10 to be similar to the control plants for the remainder of the trial. R0.12 increased its NCPA levels at 12 and 24 hpi, compared to the control plants, but levels returned to the constitutive state by 96 hpi. At 96 hpi, R0.10 had the highest NCPA levels; R0.12 had significantly lower amounts than R0.10, whereas R0.06 significantly differed from both R0.10 and R0.12 by having the least NCPA. Also noteworthy of mention is that R0.06 had the lowest NCPA levels (0.53 mg gallic acid equivalent per gram dry weight) at this time

point (96 hpi) when compared to all the rootstocks at all the sampling times. This value was significantly different from the control plants (2.29 mg gallic acid equivalent per gram dry weight).

Total soluble phenolic acids (TSPA)

The combined values for each of the targeted phenolics reflected that of the value obtained from the crude extract, although some phenolics were likely lost during treatment of individual fractions. Although there was a negative trend in the slope of TSPA expression over time in resistant R0.06 roots, levels in this rootstock did not differ significantly over the extent of the trial period (Figure 3.9). R0.06 roots also had the lowest mean TSPA concentration at each sampling point. R0.10 showed a similar pattern, where a significantly lower amount of TSPA was detected at 12, 24 and 96 hpi compared to 0 and 3 hpi. On the contrary, the susceptible R0.12 rootstock had a rapid increase in TSPA production as soon as 3 hpi. These levels gradually decreased, as a significantly lower amount was detected at 12 hpi, a slightly higher amount at 24 hpi, but significantly lower again at 96 hpi, although this value was still significantly more than that found in control plants of R0.12. TSPA in R0.06 R0.10 did not differ significantly at 96 hpi. However, the 8.14 mg gallic acid equivalent per gram dry weight TSPA in R0.12 was significantly higher than the 3.79 and 4.94 mg gallic acid equivalent per gram dry weight of R0.06 and R0.10 respectively at 96 hpi.

Enzyme assays

Fluorescent microscopy images confirmed that the inoculation method was successful. *Phytophthora cinnamomi* – like structures could be observed on the surface of all three avocado rootstocks inoculated with zoospores 12 hpi (Figure 3.10).

Ascorbate peroxidase

No differences in APX activity could be detected over the time course of the experiment within and between each of the resistant rootstocks R0.06 and R0.10. The most PRR-susceptible rootstock, R0.12, had the highest constitutive expression of APX (Figure 3.11). A negative value was obtained at 0 hpi for R0.10, which was adjusted to 0, indicating the presence of counteracting enzymes. At 0 and 6 hpi, R0.12 had the highest APX activity, but a

significant decrease in activity was observed at 12 hpi and remained low until 72 hpi.

Guaiacol peroxidase

Constitutive GPX levels in the moderately resistant R0.10 rootstock were at least five times higher than the resistant R0.06 and susceptible R0.12 rootstocks (Figure 3.12). GPX activity decreased significantly in R0.10 at 6 and 12 hpi, increased again at 24 hpi, followed by another significant decrease at 48 hpi so that GPX levels stayed low until 72 hpi. GPX levels within R0.06 and R0.12 did not differ significantly across all sampling time points; however, at 24 and 48 hpi R0.12 had significant lower GPX activity than R0.06 and R0.10.

Catalase

Catalase showed a decrease in activity in all three rootstocks over the first 12 h after inoculation with *P. cinnamomi* (Figure 3.13). By 24 hpi catalase activity had increased significantly in the resistant R0.06 rootstock. However, at 48 hpi catalase levels in R0.06 had returned to levels comparable to early time points. In R0.10, catalase activity decreased significantly as early as 6 hpi and remained low for the duration of the experiment. Catalase in R0.12 had a significant decrease in activity at 12 hpi compared to 0 hpi, but increased at 48 and 72 hpi.

Superoxide dismutase (SOD)

SOD activity did not differ significantly during the first 6 hpi between the three rootstocks (Figure 3.14), although there was an indication of increased SOD activity as early as 6 hpi in the resistant R0.06. At 12 hpi, SOD activity in R0.06 resulted in a peak, which was significantly higher than the two more susceptible rootstocks. This was also accompanied by a decrease in SOD levels in R0.10 and R0.12. At 24 hpi a significant increase in SOD was observed in R0.10, with little change in R0.06 and R0.12. A late response was observed in R0.12 with a significant increase at 48 hpi. At 72 hpi SOD activity in all three rootstocks had returned to levels comparable to early time points.

β-1,3-glucanase

A rapid increase in glucanase activity was observed in the resistant R0.06 rootstock following inoculation with *P. cinnamomi*. Glucanase activity at 0 hpi in R0.06 was the lowest, significantly higher in the susceptible R0.12 and the highest in the moderately resistant R0.10 rootstock (4.5 U mg⁻¹ protein; Figure 3.15). Six hours after inoculation, a significant increase in activity was observed in the resistant R0.06, as opposed to a significant decrease in R0.10 and R0.12. At 12 hpi, glucanase levels in R0.10 and R0.12 remained low, but a significant increase took place in R0.06 so that glucanase activity in this rootstock was significantly higher than R0.10 and R0.12. At 24 hpi, R0.10 had significant higher levels, but glucanase in R0.06 had decreased significantly and did not differ from R0.12 at this time point. R0.12 had similar glucanase levels at 24 hpi to samples obtained at 0 hpi, but this rootstock showed little variation for the remainder of the experiment by remaining constant. At 48 hpi, no significant differences were detected between the three rootstocks, as R0.10 had undergone a significant decrease in activity from 24 to 48 hpi. *β-1,3-glucanase* activity in R0.06 was significantly decreased at 72 hpi so that levels at this time point did not differ from control samples.

Discussion

This study made a significant contribution to understanding the complex interaction between *P. cinnamomi* and its host *P. americana*. The most significant finding in this study was that the resistant R0.06 initiated an early defense response by a rapid increase in glucanase and SOD production. Phenolic acids were not associated with resistance in these rootstocks. Levels of the five defense-related enzymes that were assayed in this study, APX, GPX, catalase, SOD and *β-1,3-glucanase*, were different in the three rootstocks that were tested. The findings from this study provide useful insights into the responses of avocado to this pathogen.

Increased concentrations of *β-1,3-glucanase* were evident in the resistant R0.06 rootstock during the first 12 hpi. The moderately resistant rootstock, R0.10, also produced significantly higher amounts of glucanases compared to R0.12, the more susceptible clone,

at 0 hpi and 24 hpi. These results are in agreement with studies on other host-pathogen interaction systems. For instance, after inoculating various *Vitis* spp. ranging in susceptibility with the fungus *Uncinula necator* (Schw.) Burr., a positive correlation between amounts of β -1,3-glucanase activity and degree of resistance was made (Giannakis *et al.*, 1998; Suo & Leung, 2001). In another study, the fungus *Diplocarpon rosae* Wolf, which causes black spot of roses, stimulated β -1,3-glucanase production (Suo & Leung, 2001). In the present work, the R0.06 rootstock not only rapidly increased glucanase production, but also had significantly higher β -1,3-glucanase activity at 12 hpi than the rootstocks R0.10 and R0.12. The results obtained from this study therefore suggest that an increase in glucanase enzyme during the initial stages of pathogen invasion, which includes germination of zoospores, external hyphal growth and appressoria formation (chapter 2), might influence the resistance of an avocado plant to PRR. Aveling and Rijkenberg (1991) quantified and compared the amount of encysted zoospores on four avocado rootstocks varying in resistance to *P. cinnamomi*. They found that fewer zoospores encysted on resistant rootstocks, and that fewer cysts germinated on the resistant cultivars 'Duke 7', 'G6' and 'Martin Grande' than on susceptible 'Edranol' seedlings. Similar results were obtained by Botha and Kotze (1989) on the same rootstocks. The present results are therefore consistent with the hypothesis that resistant avocado rootstocks would excrete more glucanase enzymes which may influence the establishment of the pathogen on avocado roots.

In contrast to rootstock R0.06, β -1,3-glucanase activity was significantly decreased 6 hpi in both R0.10 and R0.12 when compared to the control plants. Pathogens secrete elicitors which cause hypersensitive-like cell death and the activation of other signaling cascades in plants. However, these plant defense reactions can also be suppressed by pathogens as well. Several *Phytophthora* spp. are able to suppress plant endo- β -1,3-glucanases by means of glucanase inhibiting proteins (GIPs; Ham *et al.*, 1991; Rose *et al.*, 2002; Yoshikawa *et al.*, 1981; Yoshikawa *et al.*, 1990). Although the presence of GIPs has not been demonstrated in *P. cinnamomi*, the present results suggest that glucanase enzymes are suppressed in susceptible rootstocks. The imminent *P. cinnamomi* genomic sequence would enable examination of this hypothesis.

An increase in SOD activity was observed in all three rootstocks, but the time when maximum levels were observed after inoculation differed among rootstocks. R0.06 had the most rapid response, with a maximum peak at 12 hpi, followed by R0.10 reaching its maximum activity at 24 hpi and lastly R0.12 only reacting at 48 hpi. This suggests either delayed pathogen recognition and consequently a slower activation of signaling cascades in the more susceptible rootstocks, or a suppression of host defense response by the pathogen. SODs catalyse the conversion of superoxide anions to hydrogen peroxide (Apel & Hirt, 2004), and therefore a higher SOD concentration would ensure higher concentrations of hydrogen peroxide. In turn, lowered catalase activity after infection (as seen in this study) would allow for higher hydrogen peroxide concentrations. A fine balance between superoxide and SOD can influence disease resistance drastically and these two molecules should be studied in conjunction with each other (Scandalios, 1993).

A decreased catalase expression profile over time was demonstrated in all three rootstocks for the first 12 hpi. It is well known that necrotrophic pathogens can induce ROS production in plants (usually via a hypersensitive response) which can cause tissue damage. In the present investigation, the decrease in catalase expression in all three rootstocks for the first 12 hpi might be due to an oxidative burst in an attempt to counter pathogen invasion. Since *P. cinnamomi* is a hemibiotroph, an oxidative burst would be expected soon after penetration since this would be the most vulnerable stage for any plant pathogen (Dickinson, 2003). The drastic increase in catalase activity observed in the R0.06 rootstock at 24 hpi could be due to host opposition to the initiation of a necrotrophic phase by the pathogen. An increase in ROS production usually commences programmed cell death (Dickinson, 2003). Enzyme trends noted in this investigation may well be the result of the host plant countering the natural infection and biology of the pathogen. If an oxidative burst occurs in avocado roots early after infection, APX and catalase would be down-regulated to ensure little or no interference with the ROS. However, prolonged exposure to ROS could be detrimental to the plant and this could also explain the increase in catalase activity in R0.06 at 24 hpi.

A decrease in expression of phenolic acids in resistant rootstocks was observed after inoculation. Phenolic data collected from orchard trees also suggests that higher concentrations of phenolic acids do not confer resistance in avocado plants against *P. cinnamomi*. Previously, Cahill and McComb (1992) correlated high amounts of soluble phenolics in *Eucalyptus marginata* Sm. and *Eucalyptus calophylla* R. Br. with resistance to *P. cinnamomi*. Results showed that these two closely related host species responded differently to infection. In the present study, field data indicated that R0.12 is susceptible to PRR. Phenolic acids (total soluble, cell wall bound, non-conjugated and glycoside bound) from R0.12 avocado plants in an orchard did not differ significantly from the more resistant rootstocks R0.10 and R0.09. Furthermore, during the mistbed inoculation trial, phenolic acid expression usually decreased in the resistant R0.06 rootstock after inoculation. A decrease in phenolic content in R0.06 was observed in ester bound-, cell wall bound-, glycoside bound-, non-conjugated- and total soluble phenolics. This trend was also observed in R0.10, but an opposite, positive trend was noticed in R0.12. García-Pineda *et al.* (2010) observed a significant decrease in total soluble phenolics in susceptible avocado seedlings 4 d after inoculation with *P. cinnamomi* mycelia. Noteworthy of mention is that R0.12 was initially selected as a potential resistant rootstock, but was later rejected based on its performance in the field. This might indicate that this rootstock possesses some resistance. Also, mycelia used for inoculation in the mistbed trial might not have been the most efficient means of activating a rapid defense response.

Beckman (2000) suggested that the location of specialised cells could determine resistance, together with the defense sequence of a host plant. Thus, in the present study the amount of phenolics may not have influenced the host plant to withstand infection directly, but the location within the tissue of these compounds. The salicylic and ethylene signaling pathways can result in phenolic production (Martinez *et al.*, 2001). Phenolic compounds also have antioxidant properties (Rice-Evans *et al.*, 1997). On the other hand, many phenolic compounds have prooxidant activity at low concentrations (Fukumoto & Mazza, 2000). Some water-soluble phenolics have also been described to have antioxidant activities against H₂O₂ (Sroka & Cisowski, 2003). Therefore, the lower amounts of phenolics correlates with the data obtained from ROS-scavenging enzyme-assays in this study. Further studies,

which combine microscopy with bioassays, might clarify the exact role of phenolic acids in avocado plants.

APX and GPX activity patterns showed a negative trend over time and in all three rootstocks tended to either decrease over time, or were expressed at constant levels. The results obtained from this study are in contrast to those of García-Pineda *et al.*, (2010), who also investigated the effects of *P. cinnamomi* on peroxidase expression in avocado plants. They found that total peroxidases increased at 2 dpi with a maximum activity at 4 dpi. In the present investigation, an increase in APX activity was observed within 6 hpi in R0.12. These differences in expression patterns between the two studies could be due to different inoculation techniques, differences in environmental factors, or plant materials which were of different origins and consequently different disease susceptibility levels.

The low and varying levels of APX and GPX detected in this study could be due to fluctuations of either a production or a degradation of hydrogen peroxide, depending on the physiological state of the plant. This, in turn might be subjected to the ability of the host plant to recognise the pathogen elicitors and consequently initiate an opposing defense response. Numerous studies have been performed on enzyme activity during fruit maturation etc, but very little is known regarding enzyme expression in avocado roots in response to *P. cinnamomi* infection. Several studies performed on peroxidases in avocado include the fruit and leaves (Bower & Nel, 1981; Chiapella *et al.*, 1995; Sharon-Raber & Kahn, 1983). In a study on roots it was shown that peroxidases are involved in the rooting process of plants (Molassiotis *et al.*, 2004). The latter is possibly due to the involvement of this enzyme in the lignin biosynthesis pathway (García-Pineda *et al.*, 2010; Koduri & Tien, 1995). A peroxidase enzyme which generates hydrogen peroxide has also been observed and characterised in *Arabidopsis thaliana* (Bindschedler *et al.*, 2006).

Data obtained from this study correlated well with one other. SOD had an opposite activity pattern to the rest of the ROS-scavenging enzymes. Since low concentrations of phenolics could be pro-oxidative by increasing H₂O₂ levels, and both catalase and peroxidases were either decreased or occurred at low amounts in the resistant R0.06, we hypothesise that

ROS plays a crucial role in countering pathogen attack. The role of increased H₂O₂ in avocado roots might contribute to increased resistance. Future studies, which combine the detection of ROS-scavenging enzymes in conjunction with their respective substrates, will clarify these hypotheses.

Conclusion

In this study, activity levels and response times of several defense-related compounds were determined in three avocado rootstocks after inoculation with *P. cinnamomi*. R0.06, a highly PRR resistant rootstock, had a greater capacity to respond rapidly after infection with *P. cinnamomi* than the moderately resistant rootstock R0.10 and susceptible R0.12 by increasing its β -1,3-glucanase and SOD activities as soon as 6 hpi. The hypothesis that phenolic acid expression would be activated sooner and in higher amounts in the resistant rootstocks is rejected, in view of the fact that phenolic concentrations were reduced in R0.06 and R0.10, but increased in the susceptible R0.12. Activities of APX and GPX, two hydrogen peroxide scavenging enzymes, did not change during the sampling window in R0.06, as basal levels were already low. R0.12 had a decrease in APX and R0.10 a decrease in GPX activity. Catalase activity also decreased in all three rootstocks during the first 12 hours after inoculation. Significant increases in SOD activity were observed at 12 hpi for R0.06 and 24 and 48 hpi for R0.10 and R0.12 respectively, suggesting a time-delay for SOD production in rootstocks less resistant to *P. cinnamomi*. These results are consistent with the phenolic data, suggesting that ROS play a vital role in defense against *P. cinnamomi* in avocado rootstocks. Results obtained from this study contribute to a greater understanding of the complex biochemical weapons in avocado that are associated with response to *P. cinnamomi*.

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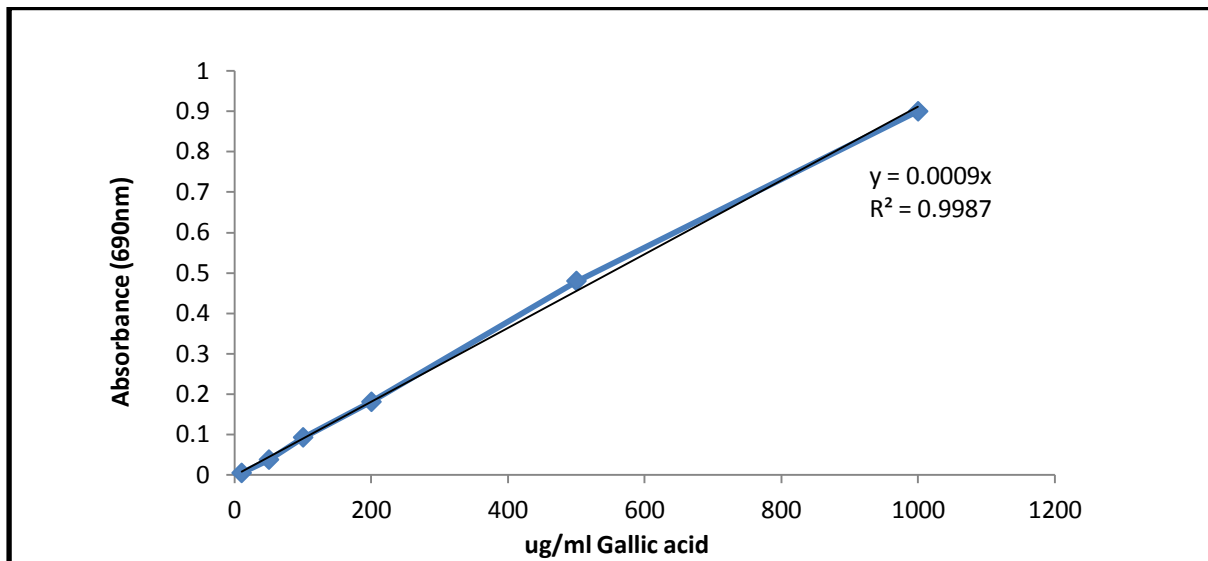


Figure 3.1. Gallic acid calibration curve used for the determination of phenolic acid content in avocado rootstocks according to the Folin-Ciocalteu method. The curve was constructed using concentrations of 10, 50, 100, 200, 500 and 1000 $\mu\text{g/ml}$ methanol. Measurements were performed in triplicate and an average was used.

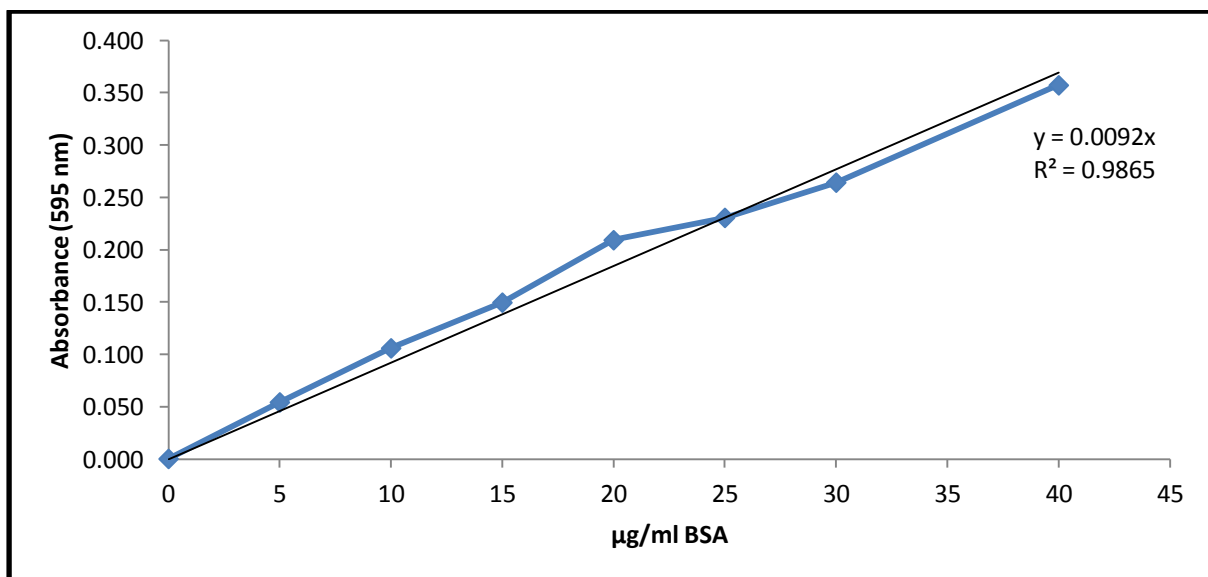


Figure 3.2. Bovine serum albumin standard curve, used to calculate total protein content in avocado roots according to the Bradford (1976) method. Reactions were performed in triplicate and an average was used to construct the curve.

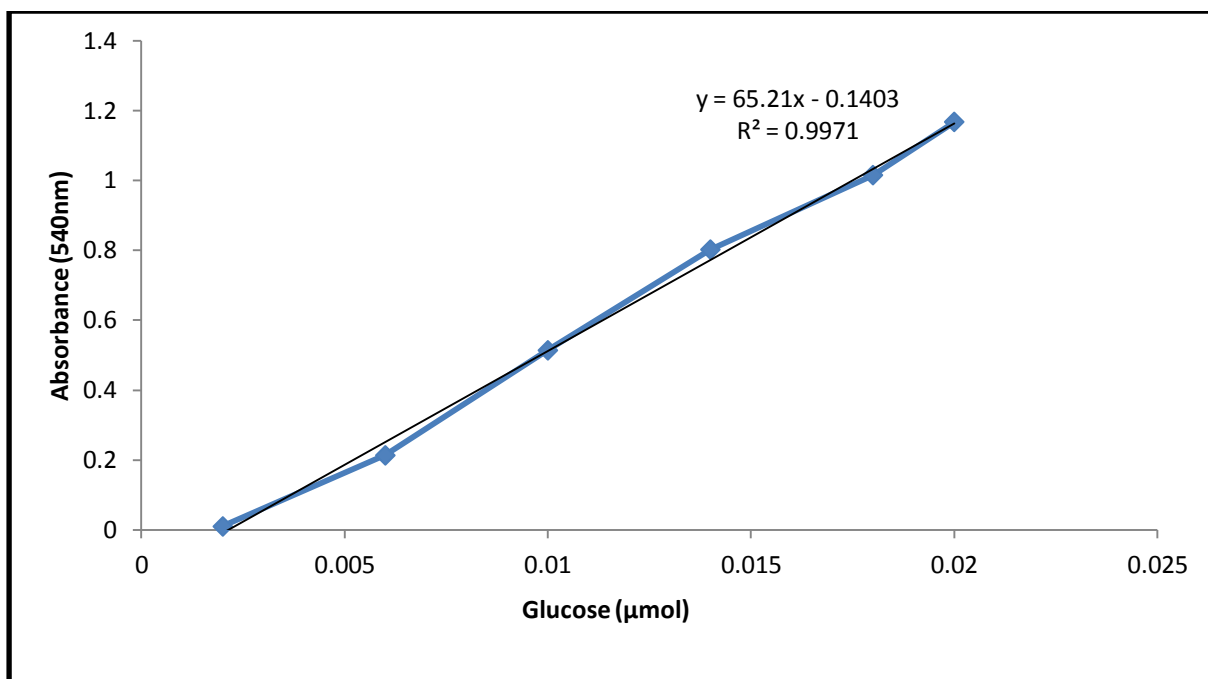


Figure 3.3. Glucose calibration curve. The curve was constructed using a 20 mM glucose solution with dilutions ranging from 0 – 10 µl. Reactions were performed in triplicate and an average was used.



Figure 3.4. Roots of three rootstocks (from left to right susceptible R0.12, moderately resistant R0.10 and resistant R0.06) 6 weeks after being inoculated with *Phytophthora cinnamomi*.

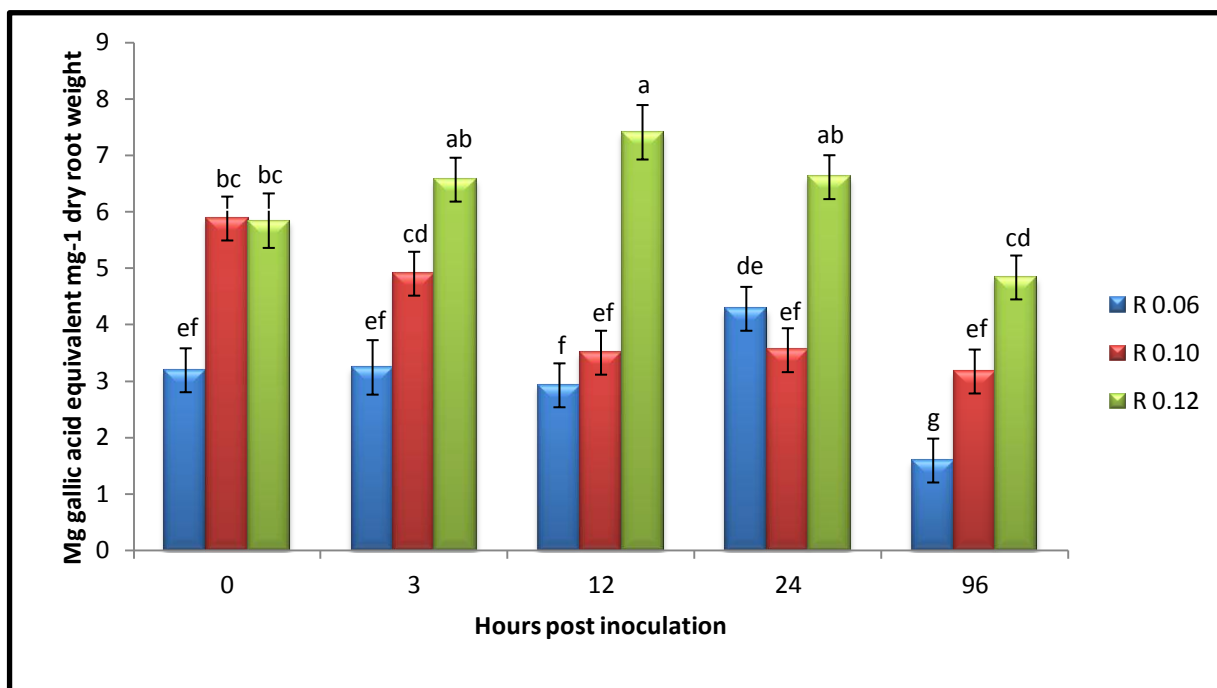


Figure 3.5. Levels of cell wall bound phenolic acids in avocado rootstocks (R0.06 – resistant, R0.10 – moderately resistant and R0.12 - susceptible) after inoculation with *Phytophthora cinnamomi* in a mistbed. Bars are means of at least three replicates \pm SE ($\alpha = 0.05$). Same letters indicate no significant difference according to Fisher’s protected pairwise test.

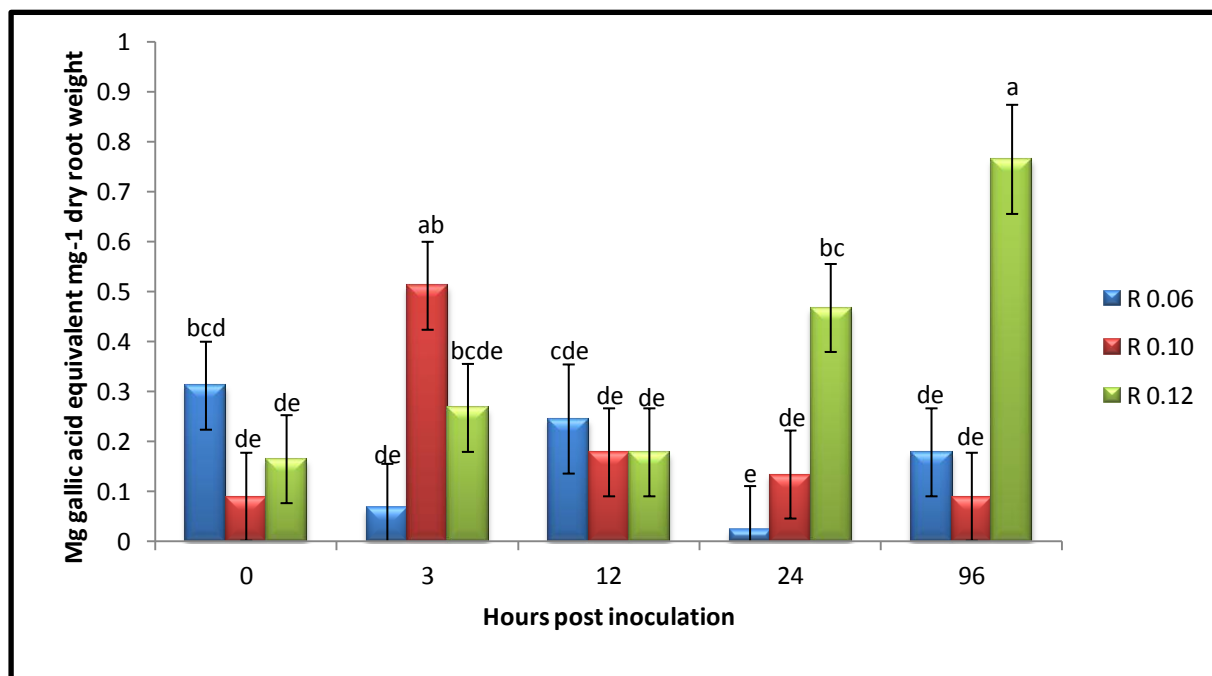


Figure 3.6. Levels of ester bound phenolic acids in avocado rootstocks (R0.06- resistant, R0.10- moderately resistant and R0.12- susceptible) after inoculation with *Phytophthora cinnamomi* in a mistbed. Bars are means of at least three replicates \pm SE ($\alpha = 0.05$). Same letters indicate no significant difference.

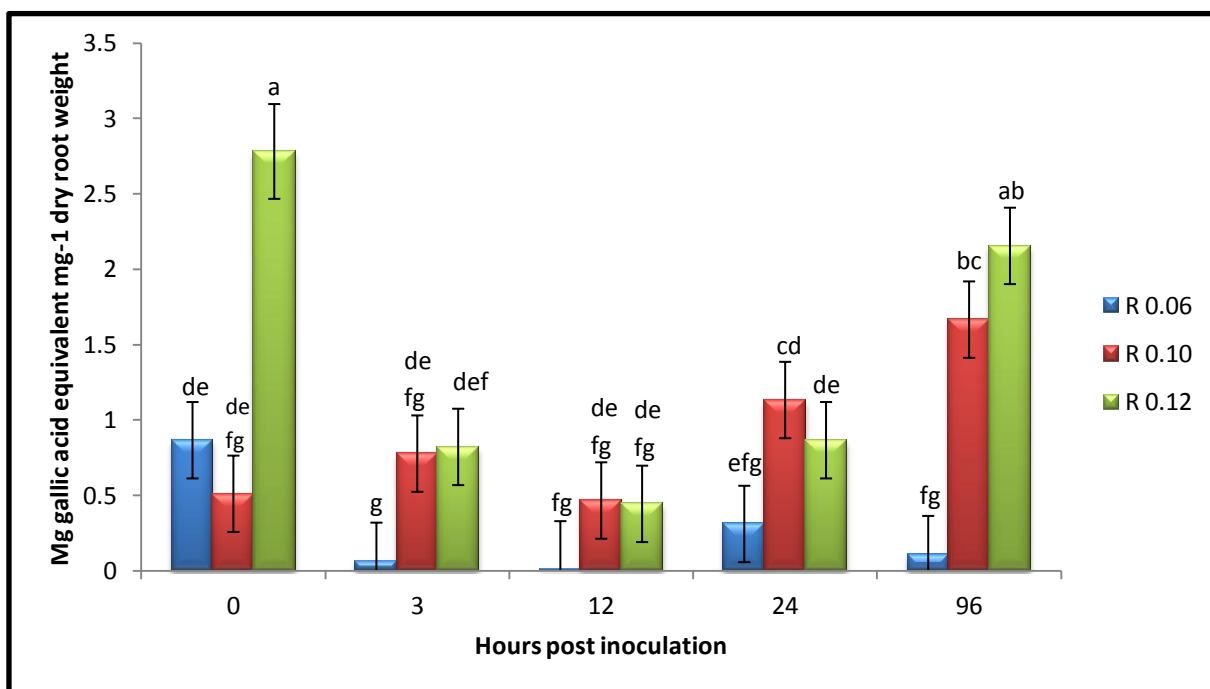


Figure 3.7. Levels of glycoside bound phenolic acids in avocado rootstocks (R0.06 – resistant, R0.10 – moderately resistant and R0.12 - susceptible) after inoculation with *Phytophthora cinnamomi* in a mistbed. Bars are means of at least three replicates \pm SE ($\alpha = 0.05$). Same letters indicate no significant difference according to Fisher’s protected pairwise test.

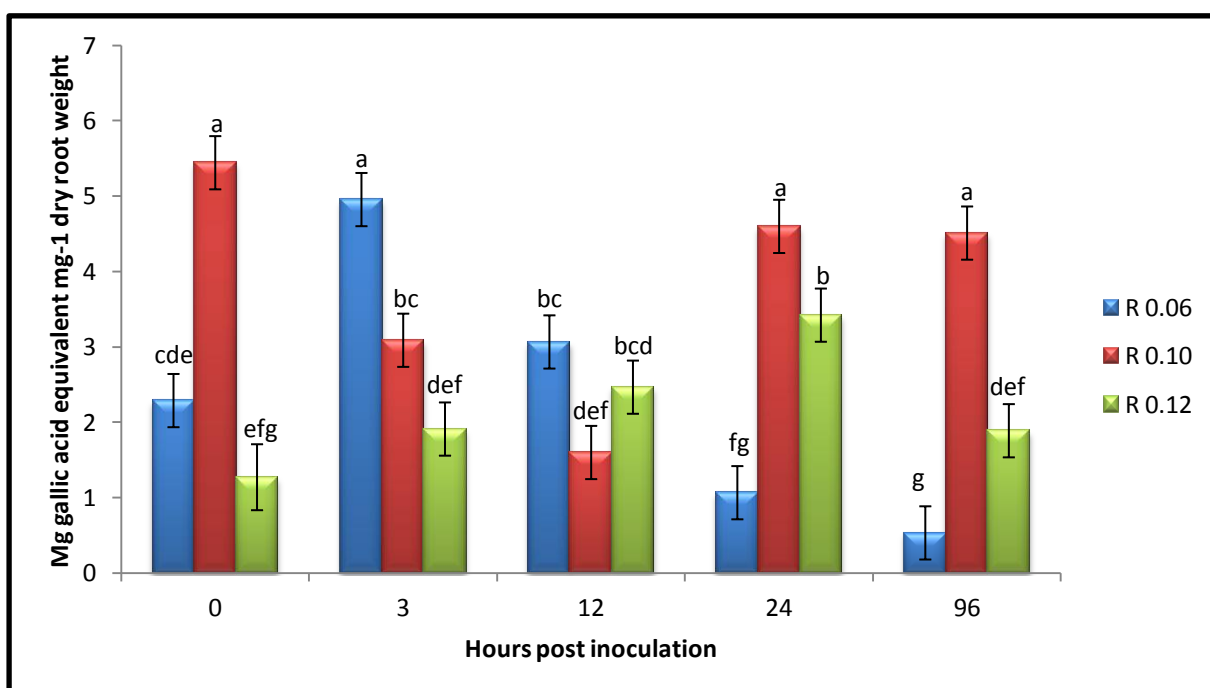


Figure 3.8. Levels of non-conjugated phenolic acids in avocado rootstocks (R0.06 – resistant, R0.10 – moderately resistant and R0.12 - susceptible) after inoculation with *Phytophthora cinnamomi* in a mistbed. Bars are means of at least three replicates \pm SE ($\alpha = 0.05$). Same letters indicate no significant difference according to Fisher’s protected pairwise test.

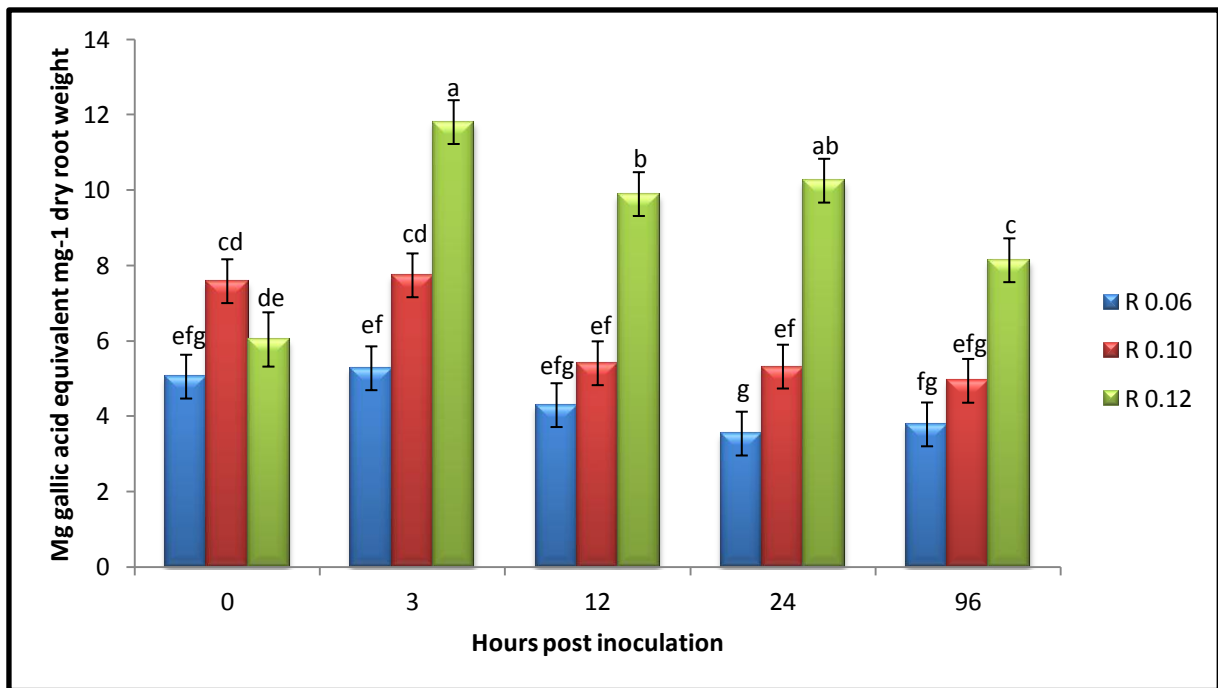


Figure 3.9. Levels of total soluble phenolic acids in avocado rootstocks (R0.06 – resistant, R0.10 – moderately resistant and R0.12 - susceptible) after inoculation with *Phytophthora cinnamomi* in a mistbed. Bars are means of at least three replicates \pm SE ($\alpha = 0.05$). Same letters indicate no significant difference according to Fisher's protected pairwise test.

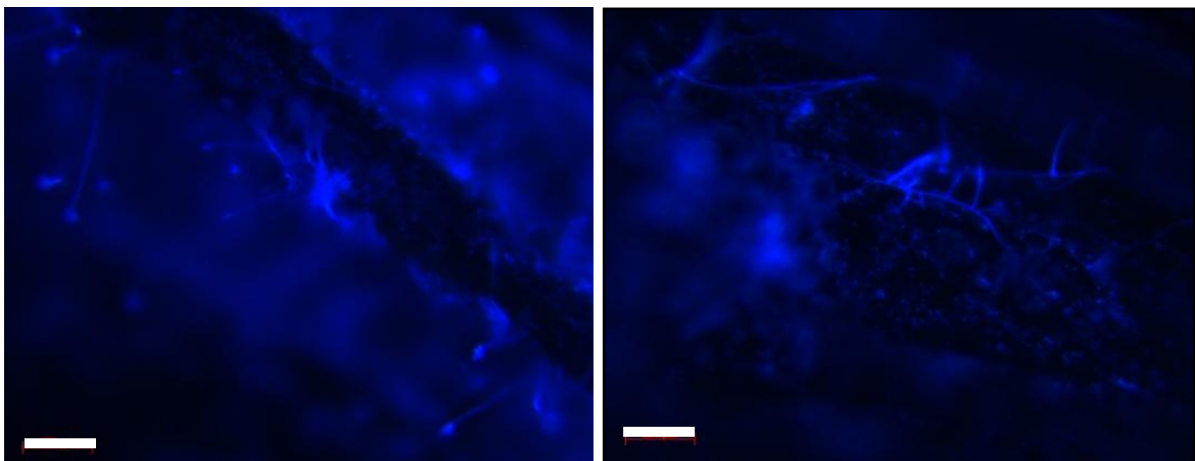


Figure 3.10. Fluorescent micrographs of germinated *Phytophthora cinnamomi* zoospore cysts and developed hyphae visible on the surface of moderately resistant R0.10 avocado roots. Scale bars = 100 μ m.

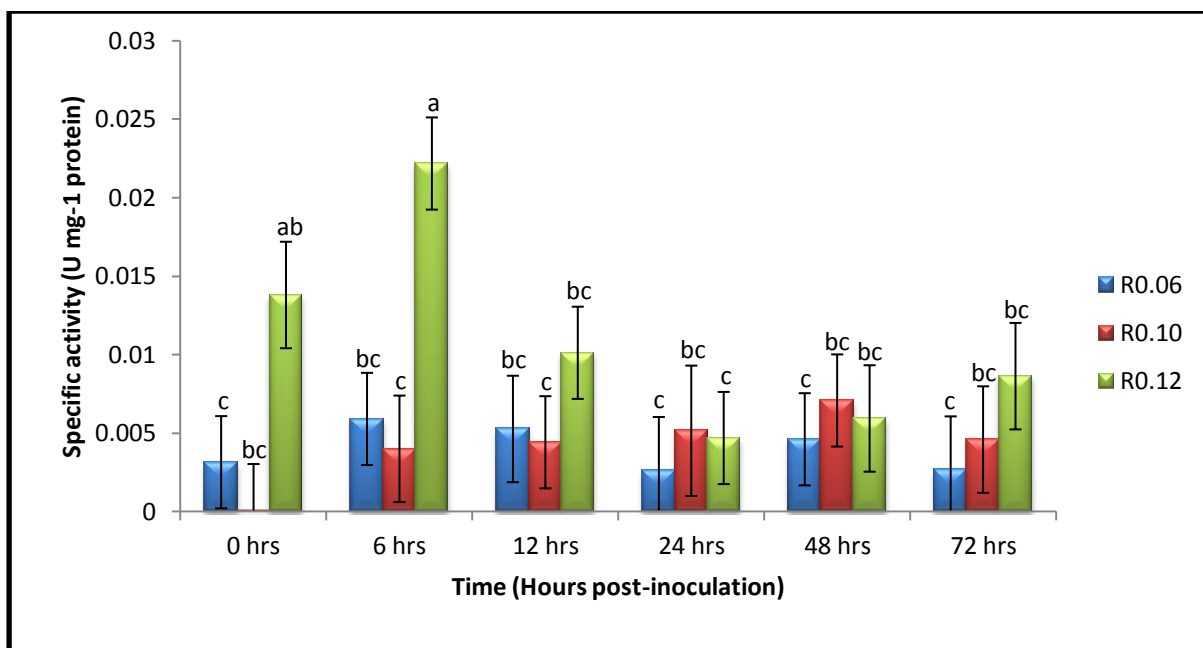


Figure 3.11. Ascorbate peroxidase activity over time in three avocado rootstocks (R0.06 – resistant, R0.10 – moderately resistant and R0.12 - susceptible) inoculated with *Phytophthora cinnamomi*. Bars are means of at least three replicates \pm SE ($\alpha = 0.05$). Same letters indicate no significant difference according to Fisher’s protected pairwise test.

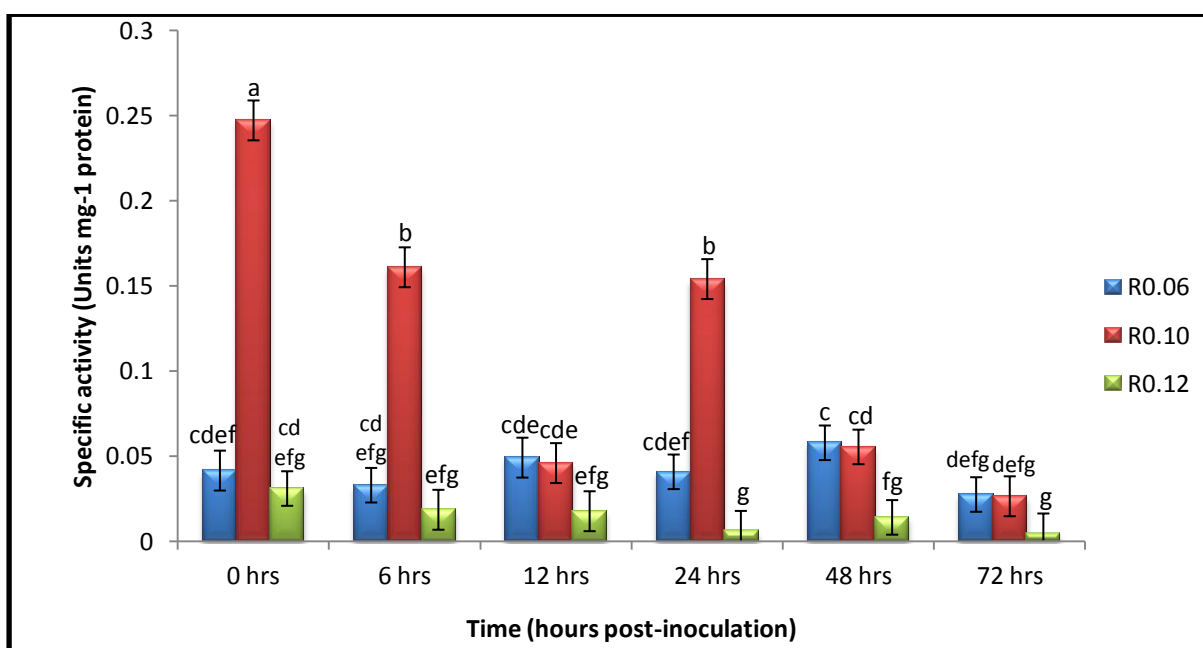


Figure 3.12. Guaiacol peroxidase activity over time in three avocado rootstocks (R0.06 – resistant, R0.10 – moderately resistant and R0.12 - susceptible) inoculated with *Phytophthora cinnamomi*. Bars are means of at least three replicates \pm SE ($\alpha = 0.05$). Same letters indicate no significant difference according to Fisher’s protected pairwise test.

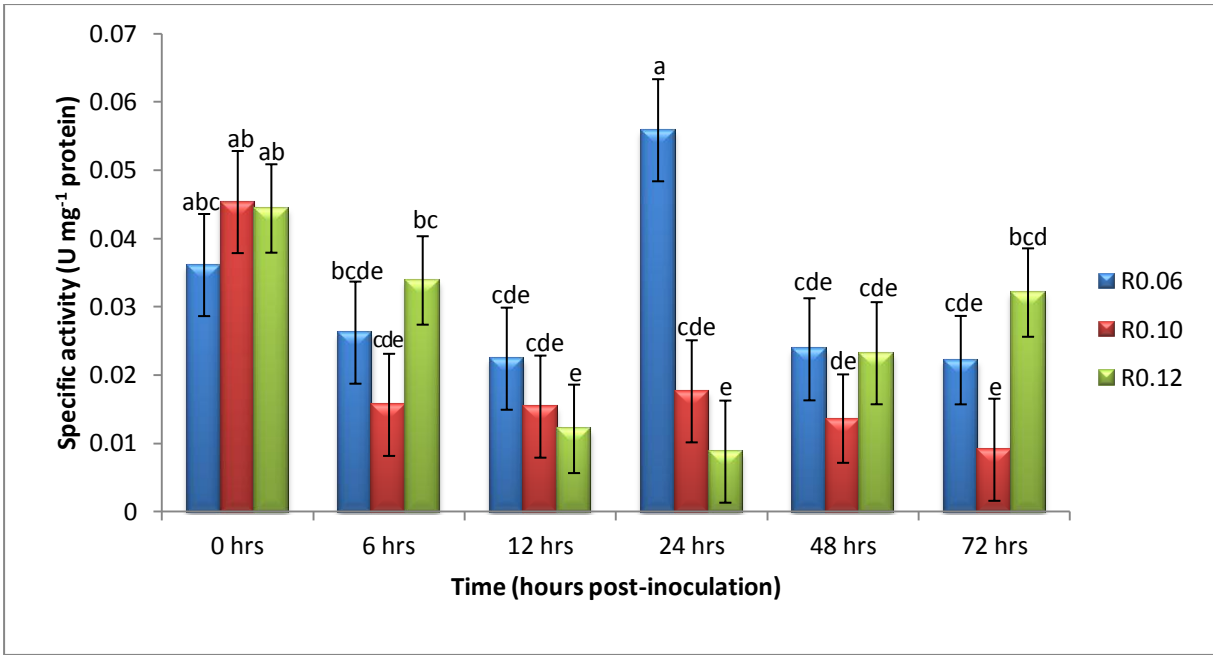


Figure 3.13. Catalase activity over time in three avocado rootstocks (R0.06 – resistant, R0.10 – moderately resistant and R0.12 - susceptible) inoculated with *Phytophthora cinnamomi*. Bars are means of at least three replicates \pm SE ($\alpha = 0.05$). Same letters indicate no significant difference according to Fisher’s protected pairwise test.

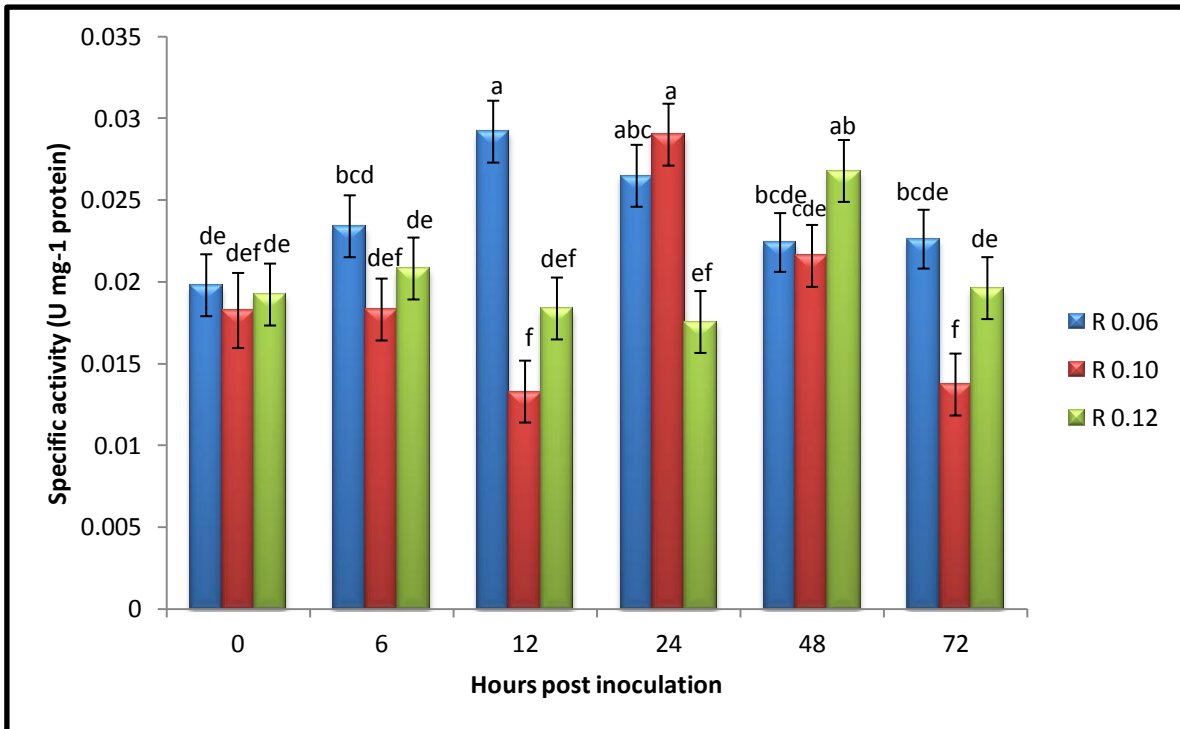


Figure 3.14. Specific activity of superoxide dismutase in three avocado rootstocks (R0.06 – resistant, R0.10 – moderately resistant and R0.12 - susceptible) after inoculation with *Phytophthora cinnamomi*. Bars are means of at least three replicates \pm SE ($\alpha = 0.05$). Same letters indicate no significant difference according to Fisher’s protected pairwise test.

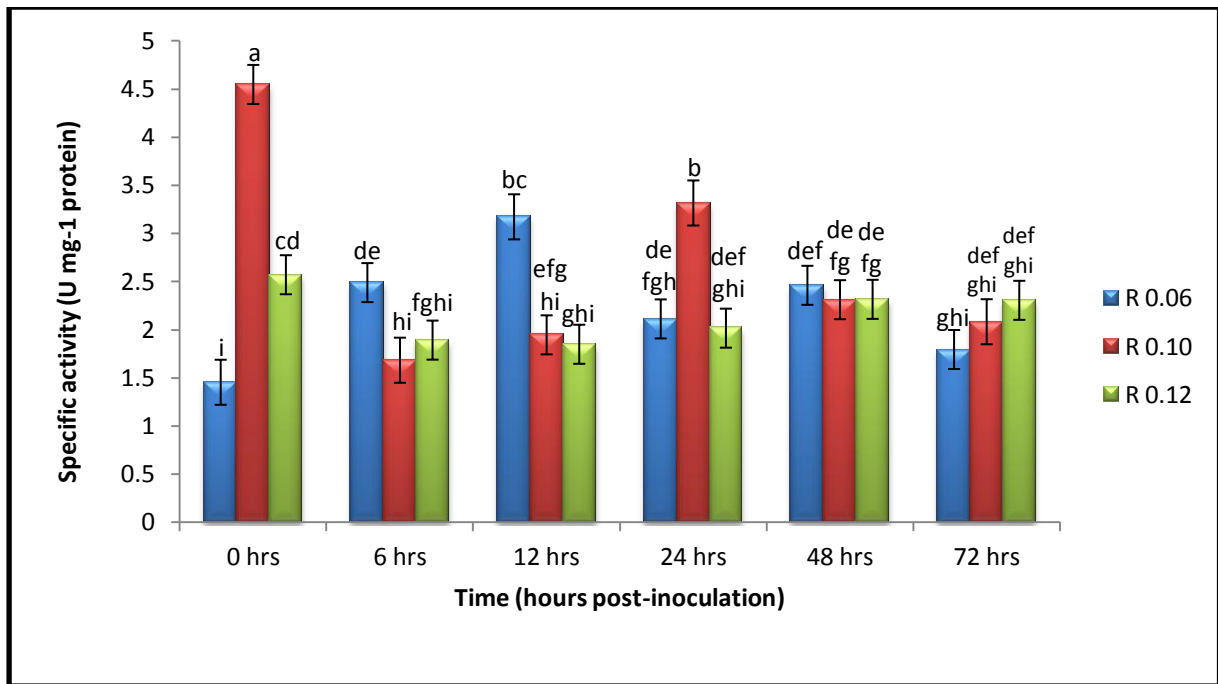


Figure 3.15. β -1,3-Glucanase activity over time in three avocado rootstocks (R0.06 – resistant, R0.10 – moderately resistant and R0.12 – susceptible) inoculated with *Phytophthora cinnamomi*. One unit (U) was defined as the amount of enzyme that produced 1 μ mol reducing sugar per min. Bars are means of at least three replicates \pm SE ($\alpha = 0.05$). Same letters indicate no significant difference according to Fisher’s protected pairwise test.

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Table 3.1. Phenolic acid expression in four avocado rootstocks (R0.10 – moderately resistant, R0.01 – resistant, R0.09 – resistant and R0.12 –susceptible), 8 years after being planted in an orchard highly infested with *Phytophthora cinnamomi* (expressed as mg gallic acid equivalent per gram dry weight). Data shown are means of at least eight trees per rootstock. Same letters in a column indicate that samples are not significantly different according to Duncan’s multiple T-test ($\alpha = 0.05$).

Rootstock	Total soluble phenolic acids	Non-conjugated phenolic acids	Glycoside-bound phenolic acids	Ester-bound phenolic acids	Cell wall-bound phenolic acids
R0.10	58.87a	7.44a	4.2a	25.33a	72.13a
R0.01	39.24b	4.05a	2.51a	20.25a	54.29b
R0.09	66.35a	6.5a	4.26a	26.11a	83.91a
R0.12	62.45a	8.3a	4.77a	24.83a	75.2a

Chapter 4

General Discussion

The avocado (*Persea americana* Mill.) is an economically important fruit crop which possesses diverse nutritional and anti-oxidative properties. Avocado originated in Mesoamerica, but is now grown in more than 60 countries. In South Africa, avocados are grown commercially in the Limpopo province, Kwa-Zulu Natal, Mpumalanga and in the Eastern Cape. *Phytophthora cinnamomi* causes Phytophthora root rot (PRR) of avocado and infection occurs predominantly by means of motile zoospores which swim chemotactically in moist soil towards its host, whereafter encystment and host penetration occurs. Infection by this devastating pathogen leads to tree decline, significantly lower yields, and often results in tree death. Consequently, high economic losses occur annually. Phosphonate trunk injections are effective in controlling PRR; however, as consumer demand trends shift towards “organic food” this measure will become increasingly unacceptable.

Plant resistance against oomycetes have been ascribed to various defense mechanisms during the past 2 decades, of which the hypersensitive response (HR) is the most widely known. Furthermore at a biochemical level, phenolic acid and reactive oxygen species (ROS) production, and increases in enzymes such peroxidases and glucanases, have all been implicated in resistance against *Phytophthora* spp. Structural defenses have also been described for various *Phytophthora*-plant interactions, where callose production has been implicated in a wide range of incompatible host-oomycete interactions, rendering this glucan polymer an attractive molecule to study.

Specific resistance mechanisms against *P. cinnamomi* have not been identified to date in avocado, which impedes the screening for specific resistance traits in breeding programmes. In this study, this need was addressed by comparing specific phenotypic traits that occur upon invasion by *P. cinnamomi* in three avocado rootstocks, namely a susceptible (R0.12), moderately resistant (R0.10) and resistant rootstock (R0.06). Morphological and anatomical differences were noted in noninoculated roots, whereafter the three rootstocks were inoculated to study the infection process, followed by histological comparisons. Finally, biochemical assays were performed to monitor the response of several defense-related

molecules after inoculation with *P. cinnamomi*, namely phenolic acids, ROS-scavenging enzymes, and β -1,3-glucanase.

Significant morphological differences were observed between the three rootstocks, as susceptible R0.12 roots were significantly thicker than those of the resistant R0.06 rootstock. Root diameter of the moderately resistant rootstock, R0.10, did not differ significantly from R0.12 or R0.06. Similar results were obtained when xylem vessel areas and root biomass were compared, as the resistant R0.06 had significantly smaller xylem vessel areas, and significantly higher root biomass than the susceptible R0.12 rootstock, but R0.10 did not differ significantly from either in either way.

Scanning electron microscopy proved useful in studying the infection process of *P. cinnamomi* on root surfaces. Terminal and intercalary appressoria formation, and indirect and direct penetration, were frequently observed. The amount of cysts present on roots was determined and compared among rootstocks and, contrary to my initial hypothesis, fewer cysts were detected per surface area on roots of the susceptible R0.12 rootstock, compared to the more resistant rootstocks. However, the germination rate of zoospore cysts was inversely proportional to the degree of resistance in rootstocks, so that cysts on resistant R0.06 roots had the lowest germination rate during the first 6 hours post inoculation (hpi).

Confocal laser scanning microscopy, in combination with Calcofluor staining proved to be invaluable in studying the progression of *P. cinnamomi* within avocado roots. Avocado roots responded by the production of callose in the resistant R0.06 and lignin in the susceptible R0.12 rootstocks, whereas both callose and lignin content increased in the moderately resistant R0.10. Lignin appeared to be ineffective in limiting pathogen growth, while callose successfully restricted colonisation by *P. cinnamomi* hyphae. Tyloses increased in susceptible R0.12 roots so that xylem vessels were completely occluded 4 days post

inoculation (dpi). Twelve dpi, R0.12 roots were completely colonised by *P. cinnamomi* hyphae, but no tyloses were observed at this time, indicating that the necrotrophic activities of *P. cinnamomi* may have degraded these structures. Hyphae were rarely observed in resistant rootstocks, which had accumulated callose depositions at the epidermis as well as in the cortex. Callose is most likely an effective barrier for *P. cinnamomi* since both callose and *P. cinnamomi* hyphae consist of β -1,3-glucan linkages, and an attempt by the pathogen to degrade callose would negatively affect the pathogen. There appeared to be a delay in response time as well as a different approach in the defense response of R0.12 against *P. cinnamomi* infection, where R0.06 responded rapidly by callose production at infected sites. These results confirm that R0.06 and R0.10 are partially resistant rootstocks, and not tolerant as previously thought, since fewer hyphae were detected as resistance ratings increased in rootstocks. The opposite was observed in R0.12, where abundant hyphae were detected 4 and 12 dpi, confirming that it is indeed susceptible.

An avocado rootstock field trial, under high *P. cinnamomi* inoculum pressure, was used to determine phenolic acid content. A mist bed was also used to inoculate plants of the three rootstocks used in this study, whereafter total phenolic acid content was measured. Results obtained in this study suggest that phenolic acids do not play a direct role in disease resistance, as higher amounts of phenolics were observed in the susceptible R0.12 rootstock when compared to R0.10 and R0.06. In fact, a downward trend in phenolic concentrations was observed in the more resistant rootstocks.

Proteins were extracted from *P. cinnamomi*-inoculated avocado roots, and the activity of several ROS-scavenging enzymes was determined. Catalase activity decreased over time in all rootstocks, and ascorbate peroxidase activity fluctuated, but was higher in the susceptible R0.12. The moderately resistant R0.10 rootstock had higher guaiacol peroxidase activity. These elevated peroxidase enzymes in R0.10 and R0.12 might explain the increase in lignin synthesis, which was observed by confocal microscopy in *P. cinnamomi*-challenged plants. Another significant result was that the resistant R0.06 rootstock had a rapid increase

in superoxide dismutase (SOD) during the first 12 hpi. A decrease in hydrogen peroxide-scavenging enzymes would ensure more hydrogen peroxide in the cytosol. An increase in SOD would also increase the amount of hydrogen peroxide, since the decomposition of superoxide anions generates hydrogen peroxide which, in turn, would aid in limiting pathogen growth. When considering all the bioassay results, it is evident that R0.06 was able to maintain higher hydrogen peroxide levels. Furthermore, phenolic acid content decreased over time in R0.06 after inoculation. Since low levels of phenolic acids can stimulate the production of hydrogen peroxide, growth of *P. cinnamomi* may be inhibited by increasing amounts of hydrogen peroxide in this rootstock.

Activity of β -1,3-glucanase rapidly increased in the resistant R0.06 rootstock. However, the presence of *P. cinnamomi* resulted in a decrease in glucanase activity in the more susceptible rootstocks, R0.10 and R0.12. This suggests suppression of β -1,3-glucanase by the pathogen, since *Phytophthora* spp. are known to produce glucanase inhibiting proteins. If suppression of this defense response occurs, it is unclear how glucanase activity in the resistant R0.06 rootstock remained unaffected. Perhaps production rates for β -1,3-glucanase in R0.06 exceeded those for suppression by the pathogen. Zoospore cysts germinated at a lower rate in resistant rootstocks. β -1,3-glucanase activity correlated with the germination rate of cysts in this study, and β -1,3-glucanase has previously been shown to inhibit cyst germination. In future studies, additional samples could be taken between 0 and 6 hpi to study cyst germination rate in conjunction with glucanase production. Study would also be warranted of the interaction between glucanase inhibiting proteins and glucanases.

In this study, we have demonstrated that PRR resistance in avocado is associated with a variety of mechanisms, and that resistance is in all likelihood conferred by a multitude of structural and biochemical responses. Firstly, the growth rate of avocado roots can influence tree health significantly, and fast root growth or regeneration rate is essential to maintain a healthy canopy. Secondly, the ability of resistant rootstocks to suppress

germination of *P. cinnamomi* zoospore cysts could enhance resistance. Thirdly, this study showed that resistant rootstocks produce more callose in response to infection, which is an effective structural barrier to restrict pathogen growth. β -1,3-glucanase was also implicated in resistance against *P. cinnamomi*. Lastly, the biochemical assays indicate that elevated hydrogen peroxide levels occur in the resistant R0.06 rootstock, as it responded quicker to infection than the more susceptible rootstocks. In total, these results contribute significantly towards understanding the complex interactions between *P. cinnamomi* and avocado rootstocks. Screening for resistant rootstocks could be based on the following three factors: i) the amount of *P. cinnamomi* hyphae that colonise avocado roots, using Calcofluor staining in combination with confocal (or light) microscopy; ii) the presence, amount and timing of callose production after pathogen challenge; and iii) assessment of β -1,3-glucanase levels. Although ROS-scavenging enzymes could also be assessed, their utility in screening assays is doubtful. Studies which compare these factors in a wider range of rootstocks are needed, as they would indicate the general role and extent to which these factors may play in resistance of avocado to this important disease.