The early physiological responses of three avocado (*Persea americana* Mill.) rootstocks to infection with *Phytophthora cinnamomi* Rands

by

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Declaration

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

Signed 

Abstract

Avocado is a globally significant fruit tree crop. However, Phytophthora root rot, caused by *Phytophthora cinnamomi* (*Pc*), is the primary limitation to production in most countries. This soil borne oomycete pathogen infects and damages avocado tree root systems, leading to canopy decline and reduced yields. The disease can be managed successfully in an integrated manner, but not totally eliminated. An important aspect of integrated control that could potentially provide a lasting solution is the use of *Pc*-resistant rootstocks. Although selection of these rootstocks is time-consuming, better understanding of the host-pathogen interaction may aid in defence marker identification which could enhance selection efficiency.

This study used *Pc*-susceptible and resistant rootstocks to examine the histological and/ or quantitative biochemical differences in callose, lignin, reactive oxygen species (ROS), antioxidants, phenolics, tyloses and gums in response to infection. This was the first quantitative study of callose and lignin deposition in R0.38, R0.06 and R0.09, and the first time *Pc*-resistance mechanisms were examined in R0.38.

Notably, the early pervasive growth of *Pc* into the stele of R0.38 concurred with its susceptibility to *Pc*. Although *Pc*-susceptible R0.38 produced more callose than *Pc*-resistant R0.06 at the earliest time point of 6 hours post inoculation (hpi), *Pc*-resistant R0.09 produced the most callose in the entire trial at the proposed biotrophic to necrotrophic switch of 12 hpi in *Pc*. This response in R0.09 is a potential biochemical resistance marker to *Pc*. There were no significant increases in lignin deposition in response to infection, but resistant rootstocks showed a significant decrease in lignin at 24 hpi, which may have been due to degradation by *Pc*. Hence the study of lignin

is unlikely to aid defence marker identification. There were no significant changes in ROS production, possibly due to assay insensitivity, but all rootstocks did show significant changes in antioxidant production. This suggests that there may have been small changes in ROS, which could have been linked to defence signalling. Phenolics and gums were produced in greater abundance and earlier in R0.38, as compared to the resistant rootstocks, and hence they were not associated with resistance to *Pc* but possibly susceptibility. Therefore they are not useful in the search for resistance markers. The role of tyloses in defence against *Pc* was inconclusive, as tyloses were observed in both R0.38 and R0.09 at the same time point of 12 hpi.

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List of Abbreviations and Symbols

%	percent
µ	micro-
ºC	degrees Celsius
3-D	three dimensional
ABS	absorbance units
ANOVA	analysis of variance
Ar	artefact
ASBVd	avocado sunblotch viroid
ATP	adenosine triphosphate
B	boron
Bl	cell wall thickening with callose
BNS	biotrophic to necrotrophic switch
Br	breakage
C	carbon
C.V.	critical value
Ca	calcium
$CaCO_3$	calcium carbonate
CICR	calcium induced calcium release
Co	cortex
CoA	coenzyme A
CPK5/26	calcium dependent kinase
CTAB	hexadecyltrimethylammonium bromide
Cy	cytoplasmic accumulation of phenolics
df	degrees of freedom

DiP	dirigent proteins
DNA	deoxyribonucleic acid
En	endodermis
Ep	epidermis
ETI	effector triggered immunity
ETS	effector triggered susceptibility
FAD	flavine adenine dinucleotide
Fr	fragments
FRAP	ferric reducing ability of plasma
g	gram
GKB4	Groenkloof block four
GSL 5	glucan synthase like five
Gu	gum
H	Hydrogen
H_2O_2	hydrogen peroxide
Ha	haustoria like structures
ha	hectare
HB5	Hazyview block five
HCl	hydrochloride or hydrochloric acid
hpi	hours post inoculation
HR	hypersensitive response
HSD	honestly significant difference
Hy	hypodermis
K	potassium
L	litre

l	litre
m	metre
m	milli-
M	molar
MAMP	microbial associated molecular pattern
MDA	malondialdehyde
$MgCl_2$	magnesium chloride
n	nano-
N	nitrogen
NaCl	sodium chloride
NADPH	reduced nicotinamide adenine dinucleotide
NaOH	sodium hydroxide
NARPH	nystatin, ampicillin, rifampicin, pentachloronitrobenzene and hymexazol
NLR	leucine rich repeat receptor
$O2^-$	superoxide anion
O_2^{2-}	peroxide ion
OH	hydroxyl group
OH^-	hydroxyl ion
$OH^\cdot$	hydroxide radical
P	phosphorous
Pc	*Phytophthora cinnamomi*
PAL	phenylalanine ammonia lyase
PAMP	pathogen associated molecular pattern
Pc	*Pc* hyphae
PCD	programmed cell death

PCR	polymerase chain reaction
PD	plasmodesmata
PDA	potato dextrose agar
Pe	perforation plate
Ph	phloem
Phr	phragmoplastin
Pi	pith
PMR4	powdery mildew resistant 4
PR proteins	pathogenesis related proteins
PRR	*Phytophthora* root rot
PRS	Pathogen recognition receptors
PTI	PAMP triggered immunity
PTS	pathogen triggered susceptibility
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
RBOH	respiratory burst oxidase homolog
rcf	relative centrifugal force
Re	cell wall thickening with lignin
RFU	relative fluorescence units
RICR	ROS induced calcium release
Rop	rho-like guanosine triphosphatase
ROS	reactive oxygen species
rpm	revolutions per minute
SAR	systemic acquired resistance
Sc	sclereid

SD	standard deviation
Sg	starch grain
Sig.	significance
SOD	superoxide dismutase
Sp	intercellular spaces
SuSy	sucrose synthase
Taq	*Thermus aquaticus*
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TE-buffer	Tris and ethylenediaminetetraacetate buffer
TPTZ	2, 4, 6-Tris (2-pyridyl)-s-triazine (TPTZ)
TROLOX	(±)-6-Hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid
Ty	tyloses
U	unit
UDP-G	uridine diphosphate glucose
UGT1	UDP-G transferase
UV	ultra-violet
Xy	xylem
Zn	zinc
α	alpha
β	beta

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Chapter 1

Introduction

Avocado (*Persea americana* Mill.) is a commercially important, non-native fruit tree crop in South Africa. However, both biotic and abiotic stresses reduce production volumes for local and export markets, which if mitigated could provide added profit for the country (Donkin, 2007). The main stress currently affecting avocado production in South Africa is Phytophthora root rot (Wolstenholme, 2010, Blakey and Wolstenholme, 2014). The disease is caused by the notorious oomycete pathogen *Phytophthora cinnamomi* Rands (*Pc*), which is a threat to multiple agricultural and native species (Hardham, 2005, Hardham, 2009, Kamoun *et al.*, 2015). The disease attacks the avocado root system and interferes with water and nutrient uptake, which leads to canopy decline, decreased yields and eventual death of susceptible trees (Oßwald *et al.*, 2014). Integrated management strategies cost time and resources and whilst they do provide control, they are not a cure. Of the available control strategies, the use of tolerant and resistant avocado rootstocks is effective and convenient, but selection is a lengthy process and the mechanisms of polygenic tolerance and resistance are not yet fully understood (Ben-Ya'acov and Michelson, 1995, Wolstenholme, 2003, Wolstenholme, 2010, Mahomed and van den Berg, 2011). Better understanding may aid in the development of resistance markers to streamline rootstock selection and increase production revenue by further reducing infection. Clues to the resistance mechanisms can be found from studies on resistant avocado rootstocks infected with *Pc* as well as those conducted on other plant species infected with fungal or oomycete pathogens (Cahill and Weste 1983, Phillips *et al.*, 1987, Cahill and M^{c}Comb, 1992, van den Berg *et al.*, 2018).

The main interests of this study were the production of callose and lignin as barriers to infection, the accumulation of phenolics, the production of gum and tyloses in xylem

vessels and the production of reactive oxygen species (ROS) and associated antioxidants in the avocado response to *Pc* infection.

Callose is a versatile biopolymer of glucose which is produced inter alia, in response to biotic and abiotic stresses. During plant defence, it commonly forms a barrier against pathogen entry by the thickening of plant cell walls (Ellinger and Voigt, 2014). Cahill and Weste (1983) compared callose deposition in thirteen species of plants when infected with *Pc* and they found that almost all of the tolerant or resistant species produced callose as a defence response, whereas none of the susceptible species produced callose. Six hours after inoculating avocado rootstocks with *Pc*, callose deposition was observed in a resistant R0.06 rootstock, but not a susceptible R0.12 (van den Berg *et al.*, 2018).

Lignin is a waterproof polyphenolic biopolymer also produced, inter alia, in response to biotic and abiotic stresses. In plant defence it also thickens cell walls as a barrier against pathogen entry (Barros *et al.*, 2015). Cahill and M^{c}Comb (1992) infected eucalypts with *Pc* and found more lignin deposition in the resistant eucalypt than the susceptible. Studies done on *Pc*-infected avocado rootstocks showed lignin deposition at 6 hours post inoculation (hpi) associated with a susceptible R0.12 rootstock rather than a resistant R0.06 (van den Berg *et al.*, 2018).

Phenolics are a diverse family of aromatic compounds containing the phenol functional group. Some phenolics, such as tannins, can be produced in plant defence against pathogens (Taiz and Zeiger, 2010). Cahill *et al.* (1993) showed that lines of *Eucalyptus marginata* resistant to *Pc* produced more phenolics than susceptible lines after

infection. Most studies on avocado have associated the production of phenolics with resistance. For example, a study performed on moderately field resistant ‘Duke 7’ avocado rootstocks found the accumulation of phenolics in cells in response to infection with *Pc* (Phillips *et al.*, 1987).

Tyloses are growths of parenchyma cells into xylem vessels that block sap flow and can be associated with gum or phenolics. Gum is a polysaccharide and pectin based parenchymal secretion that functions to block vascular tissue in plants. The blocking of xylem vessels with tyloses and gums is normally associated with plant ageing, but can also occur in response to an embolism or infection (De Micco *et al.*, 2016). Parke *et al.* (2009) found an increase in tyloses in susceptible tan oak infected with *P. ramorum*. A study done on avocado rootstocks infected with *Pc* showed that a susceptible R0.12 produced tyloses earlier at 6 hpi whereas a resistant R0.06 showed later production at 12 hpi (van den Berg *et al.*, 2018).

The production of ROS results either from metabolism, or from stress, or as a plant defence response. ROS can play a critical role in defence signalling locally and distally within the whole plant, in response to infection. High concentrations of ROS are also capable of causing direct damage to pathogens (Torres, 2010, Baxter *et al.*, 2014). Levels of ROS and oxidative stress in plants are controlled by antioxidants, which are classed as either chemical or enzymatic (Apel and Hirt, 2004, Mittler *et al.*, 2004). Allardyce (2011) showed that resistant maize plants produced ROS early in response to *Pc* infection.

1.1 Problem Statement

Currently the avocado industry relies on very few productive rootstocks that are tolerant or resistant (see Section 2.2.4 for definitions) to *Pc*. The industry requires more productive rootstocks of increased tolerance or resistance to further reduce production losses and increase revenue. Rootstock breeding and selection is a time-consuming process and resistance markers could help accelerate the process of resistant rootstock selection, although productivity would still need to be assessed. Elucidation of the early defence response of resistant rootstocks may aid in the identification of resistance markers, which could potentially lead to more efficient resistant rootstock selection.

1.2 Hypotheses

1. Early cell wall thickening by callose deposition to block pathogen ingress is associated with resistance in avocado rootstocks infected with *Pc*. Such rootstocks may produce callose earlier or in greater quantities than susceptible rootstocks when infected with *Pc*.
2. Early cell wall thickening by lignin deposition is associated with susceptibility in avocado rootstocks infected with *Pc*. Such rootstocks may produce lignin earlier or in greater quantities than resistant rootstocks when infected with *Pc*.
3. As ROS are critical to defence signalling in response to infection, an earlier or larger amount of ROS would be produced in resistant avocado rootstocks compared to susceptible rootstocks during *Pc* infection.
4. As antioxidants are necessary to control levels of ROS, resistant avocado rootstocks may produce more antioxidants than susceptible rootstocks during *Pc* infection.

5. As phenolic accumulation within cells was previously seen in a moderately field resistant rootstock in response to infection, *Pc* infected resistant rootstocks may produce more phenolics and at earlier time points than susceptible rootstocks.
6. As the formation of tyloses were previously observed earlier in a susceptible rootstock in response to infection, and tyloses can be associated with gum deposition in xylem vessels, *Pc* infected susceptible rootstocks may produce tyloses and gum earlier or more tyloses and gum than resistant rootstocks.

1.3 Aims

To compare the early infection response of resistant and susceptible avocado rootstocks infected with *Pc*.

1.4 Objectives

1. To infect resistant and susceptible rootstocks with *Pc* and to harvest root tissue at 6, 9, 12 and 24 hours after infection.
2. To qualitatively observe the location and relative amount of cell wall thickening by callose and lignin, the accumulation of phenolics in cells and tylosis and gum formation in xylem vessels due to *Pc* infection, in harvested root sections, by the use of confocal microscopy.
3. To quantitatively determine the amount of callose and lignin present and the relative amount of ROS and total antioxidants, due to *Pc* infection, in harvested root tissue by the use of absorbance or fluorescence based microplate assays.

This is the first time the R0.38 rootstock has been examined using confocal microscopy and the first time that quantitative measurements for callose, lignin, ROS

and total antioxidants have been performed on R0.38, R0.09 and R0.06 avocado rootstocks.

1.5 Dissertation Outline

Chapter 2 is entitled “The early physiological response of avocado rootstocks to infection with *Phytophthora cinnamomi*” and presents a synthesis of literature, which introduces Phytophthora root rot of avocado and then focuses on plant defence mechanisms against pathogen invasion and their relevance to root rot of avocado.

Chapter 3 examines the histochemical differences in the response of two resistant (R0.06 and R0.09) and one susceptible (R0.38) avocado rootstock, to infection with *Pc*, by using confocal microscopy.

Chapter 4 examines the quantitative differences in the response of two resistant (R0.06 and R0.09) and one susceptible (R0.38) avocado rootstock to infection with *Pc* using microplate assays.

Chapter 5 is a general discussion of the results of the previous two chapters and the inferences drawn from them.

Chapter 6 is an appendix containing explanatory statistics from the quantitative assays in chapter 4.

1.6 References

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Chapter 2

The early physiological response of avocado rootstocks to infection with *Phytophthora cinnamomi*

2.1 Avocado

2.1.1 Origin

In the kingdom of Plantae, within the clades of Angiospermae and Magnoliids, is the Lauraceae family. Therein lies the genus and subgenus *Persea*, which contain the commercial avocado (*Persea americana* Mill.). The avocado is a genetically diverse species that has three races and each race is named for its proposed region of origin: the Mexican (*P. americana* var. *drymifolia*), Guatemalan (*P. americana* var. *guatemalensis*) and West Indian (*P. americana* var. *americana*) (Bergh and Ellstrand, 1986). The appearance of the avocado tree varies in height and canopy morphology. The root system tends to be shallow with feeder roots that seek out soil rich in organic material. The fruit has a variable skin colour and texture, and contains a large pit. The nutritious flesh, which is rich in oils, vitamins and minerals, made it attractive for domestication and cultivation (Dreher and Davenport, 2013, Schaffer *et al.*, 2013). The domestication of the avocado began in Mesoamerica possibly by 6400 BC (Smith, 1966). During the colonisation of South America, which began in 1492, the Spanish found local tribes cultivating the avocado. The Aztec word “Ahucatl” for avocado was adopted by the Spanish as aguacate, which finally became the word avocado in English. The Spanish acquired the avocado, which then spread to the rest of the world (Storey *et al.*, 1986, Columbus and De Las Casas, 1991).

The Dutch are said to have brought the avocado to South Africa between 1650 and 1700 (Witney, 2002). By the late 1800s, trees grown from seeds of the West Indian race, which is more suited to grow in milder areas, were noted on the east coast of South Africa. In 1925 Mexican and Guatemalan races and their hybrids, which tolerant to colder conditions at higher elevations, were imported into South Africa as budded

trees. Amongst these was ‘Fuerte’, which had already proven itself as a suitable cultivar in California and subsequently became popular with growers in South Africa (Blatt, 1931). Modern cultivars used by avocado growers are a mix of the three races and are therefore a blend of the race characteristics (Table 2.1) (Bergh, 1992).

2.1.2 Production

Notable cultivars produced in South Africa (which are grafted onto rootstocks) include ‘Carmen’, ‘Gem’ ‘Lamb-Hass’ and ‘Maluma’ (SAAGA, 2019, Blakey and Wolstenholme, 2014). It is estimated that over 17 500 ha of land are under avocado production, with these areas being mainly found in the warmer north-east of the country in the Limpopo and Mpumalanga provinces, but also in cooler areas nearer to the coast in the KwaZulu-Natal province and the Eastern and Western Cape Provinces. In the north-east, production areas include Levubu, Tzaneen, and Nelspruit and nearer to the coast, they include Greytown, Baynesfield, Patensie and George. The avocado season runs from March to October and hotter areas produce yields earlier than cooler areas (Toerien *et al.*, 1992, Donkin, 2007, DAFF, 2017b, SAAGA, 2019). Avocados are produced for the local, export and processed markets (oil and guacamole) and the industry profits the country with job creation and revenue in local and foreign currency (DAFF, 2017b). About 170 000 tons of avocados were produced in 2018, with approximately 50 % having been exported mainly to the European Union (Donkin, 2007, Blakey and Wolstenholme, 2014, SAAGA, 2019, FAO, 2018). Global commercial avocado production is significant, with the total production volume in 2014 estimated at over 5 million tons (FAO, 2018). Fortunately, modern transportation, controlled atmosphere storage and differing production and maturity times make the global market dynamic. Hence countries compete for time-windows of low supply in

the export market to capitalise on high prices (Donkin, 2007). Notable avocado producers on the global market include Argentina, Brazil, Chile, Colombia, Israel, Kenya, Mexico, Peru, Spain and South Africa (SAAGA, 2019). Avocado production volumes have not yet reached their potential compared to other fruit that are produced in similar climates (Naamani, 2011, FAO, 2018). Volumes are currently limited by abiotic and biotic stresses and the main biotic stress is Phytophthora root rot (PRR), which is caused by the oomycete *Phytophthora cinnamomi* (*Pc*) (Ploetz, 2003, Wolstenholme, 2010).

Table 2.1: Commercial characteristics of the avocado races (Bergh, 1992).

RACE	**Mexican**	**Guatemalan**	**West Indian**
Climate	Subtropical	Subtropical	Tropical
Cold tolerance	Most	Intermediate	Least
Salinity tolerance	Least	Intermediate	Most
Alternate bearing	Less	More	Less
FLOWER			
Season	Early	Late	Early or intermediate
Bloom to maturity	5 to 7 months	10 to 18 months	6 to 8 months
FRUIT			
Size	Very small to medium	Small to large	Medium to very large
Shape	Mostly elongate	Mostly round	Variable
SKIN			
Colour	Usually purple	Black or green	Pale green to maroon
Surface	Waxy coating	Variably rough	Shiny
Thickness	Very thin	Thick	Medium
SEED			
Size ratio	Large	Often small	Large
Cavity tightness	Often loose	Tight	Often loose

2.1.3 Abiotic and Biotic Stresses

In all crops, including avocado, optimal growth is necessary to achieve high yield and good quality (Cassman, 1999). Plant stress is any exterior influence on the plant that disrupts its metabolism and hence prevents it from functioning optimally. Plant stresses can be categorised as either abiotic (non-living origin) or biotic (living origin). Plants deal with these stresses by avoidance, tolerance or acclimation. If the plant does not mitigate a stress in some way, its growth will be significantly affected and this could lead to death in severe incidences (Lichtenthaler, 1996, Lichtenthaler, 1998, Taiz and Zeiger, 2010).

Abiotic stresses include drought, flooding, mineral toxicity and salinity, extremes of temperature, high light intensity and mechanical stress (Gill *et al.*, 2016, Meena *et al.*, 2017). Mexican and Guatemalan avocado races are adapted to cool upland areas in Mesoamerica, with fair shade and high rainfall and well drained soils with a layer of mulch (Campbell and Malo, 1976, Schaffer *et al.*, 2013). Therefore, as compared to the area of avocado origin, the seasonal rainfall and hot climate in South Africa makes avocado is mostly susceptible to drought, flooding, salt stress, extremes of temperature and high light intensity (Schulze, 1947, Blakey and Wolstenholme, 2014).

Plants require water for a number of functions, which include cell turgor to maintain structure, the transport of solutes to and from the canopy, evaporative cooling and general metabolism which includes photosynthesis (Gates, 1964, Shao *et al.*, 2008a, Shao *et al.*, 2008b, Taiz and Zeiger, 2010). As drought stress is caused by a prolonged water deficit in the soil (Jaleel *et al.*, 2009), irrigation should be scheduled according to soil type, which not only prevents water deficits but also waterlogging. The use of

mulches is recommended as it keeps the soil surface moist, encourages feeder root growth for increased water uptake and can improve the structure of the soil for increased drainage (Wolstenholme *et al.*, 1997, Schaffer *et al.*, 2013). Avocado trees are also reported to use more water during fruit drop, flowering and fruit set and vegetative and root flushes. Hence the irrigation schedule should be altered accordingly during these periods (Du Plessis, 1991, Schaffer *et al.*, 2013).

Flooding is a stress that results from excess water in the soil. The excess water can saturate the soil pores for an extended period and thereby limit oxygen diffusion (hypoxia or anoxia). The lack of oxygen induces anaerobic respiration in plants, which quickly consumes carbohydrates and exhausts energy stores, resulting in the production of toxic levels of lactic acid (Inglett *et al.*, 2005, Taiz and Zeiger, 2010). In addition, flooding also favours *Pc* infection in avocado as it enhances zoospore mobility (Schaffer and Ploetz, 1989). The combined impact on avocado of flooding and *Pc* infection has been observed to be far more severe than either stress alone (Reeksting *et al.*, 2014a). Orchard sites should therefore be carefully chosen for good drainage without soil compaction or impermeable layers, but where this is unavoidable, ridges can be used to increase soil drainage if necessary (Du Plessis, 1991, Myburgh, 2017).

Salt stress in plants results from decreased water uptake due to increased soil osmotic potential resulting from the accumulation of salts in the soil (Blaylock, 1994). Accumulation can be caused by irrigating with saline water or irrigating in arid environments with high evapotranspiration rates. Avocado is very susceptible to salt stress and can also suffer from chloride toxicity, sodium accumulation and lime

induced chlorosis (at high pH, at which iron becomes unavailable) (Wallace and North, 1953, Bernstein, 1965, Bingham *et al.*, 1968, Schaffer *et al.*, 2013). To avoid salt stress, salt-tolerant avocado rootstocks should be planted and irrigation water should be of good quality. If salt accumulation occurs, irrigation should be used to leach the salts out of the root zone (Blaylock, 1994, Ben-Ya'acov and Michelson, 1995, Crowley, 2008, Schaffer *et al.*, 2013).

Temperature stress is caused by plant exposure to temperature extremes. High temperatures can cause heat stress, which lowers the rate of photosynthesis and decreases cell membrane stability (Levitt, 1980, Los and Murata, 2004, Taiz and Zeiger, 2010). In order to avoid yield loss, avocados should not be planted in hot dry areas (Schaffer *et al.*, 2013). However, if this cannot be avoided, overhead or canopy irrigation can help to lower the tree temperature (Lomas and Mandel, 1973). In contrast, low temperatures can cause cold stress, which decreases cell membrane fluidity and decreases associated chemical reactions, thus slowing plant metabolism (Levitt, 1980, Los and Murata, 2004). Frost causes physical damage by the formation of sharp ice crystals in plant cells and by cellular dehydration (Levitt, 1980, Taiz and Zeiger, 2010). Irrigating the orchard just before the frost event occurs is beneficial, as the water can hold and store heat well, which can be re-radiated back into the orchard at night. Increasing insulation by using shade cloth over trees or allowing dense canopy growth can also mitigate the effects of frost (Businger, 1965, Malo *et al.*, 1977, Schaffer *et al.*, 2013).

High light intensity causes plant stress by photoinhibition and by producing sunscald injuries (Demmig-Adams and Adams, 1992, Agrios, 2005, Taiz and Zeiger, 2010).

Young avocado plants are particularly vulnerable and benefit from placement under shade netting and being painted with acrylic paint to reflect light. Older trees and newly pruned trees can also benefit from painting to prevent trunk sunscald (Rounds, 1956, Bender, 2012, Schaffer *et al.*, 2013).

Biotic stresses include herbivorous insects and nematodes, other plants, as well as plant pathogenic viruses, viroids, bacteria, fungi, oomycetes, protists and mycoplasmas (Buchanan *et al.*, 2000, Agrios, 2005). The number of insect pests in South Africa has grown concurrently with the size of the avocado industry. Notable pests include the green vegetable bug *Nezara viridula*, the coconut bug *Pseudotheraptus wayi*, the Natal fruit fly *Ceratitis rosa*, the heart-shaped scale *Protopulvinaria pyriformis*, the false codling moth *Thaumatotibia leucotreta* and the thrips *Heliothrips haemorrhoidalis* and *Selenothrips rubrocinctus*. All of these pests cause avocado fruit damage, which reduces sale value, especially for the export market (De Villiers and van den Berg, 1987, Dennill, 1992, Erichsen and Schoeman, 1992, Blakey and Wolstenholme, 2014, DAFF, 2017a). Thrips and other sucking insects pierce and feed on fruit, causing wounds that may allow fungal pathogens to enter. The false codling moth and fruit fly oviposit eggs under the fruit skin and hatching larvae feed on fruit tissue. Scale insects feed consume plant sap and excrete honeydew onto the fruit surface in which sooty mould grows (De Villiers and van den Berg, 1987, Dennill, 1992, Schaffer *et al.*, 2013).

Chemical control is available for insect pests, such as acephate for the coconut bug, methamidophos and monocrotophos for thrips and scale and trichlorfon for fruit fly (Robertson, 1990, Erichsen and Schoeman, 1993, Grové *et al.*, 1998, Schoeman *et*

al., 2010, Blakey and Wolstenholme, 2014). However, there is a modern impetus to use integrated pest management, which utilises all available control methods in order to limit chemical use and thereby reduce possible adverse environmental effects (Schaffer *et al.*, 2013, Blakey and Wolstenholme, 2014). Available methods include modifying the environment by the planting of trap crops and removal of host weeds, interference with reproduction and growth by using pheromone traps or growth inhibitors and biological control by the protection of natural pest enemies or encouragement of their numbers (Pedigo and Rice, 2014). Trap crops can be used to control the coconut bug and green vegetable bug by growing sunflowers or sunn hemp near avocado orchards (Schoeman, 2010, Schoeman *et al.*, 2010). The removal of bugweed, *Solanum mauritanum*, a fruit fly host, aids in control (Schwartz, 1978). The pheromone trap Trimedlure ® attracts Mediterranean and Natal fruit fly males and has been effectively used to control these species (Grové et al., 1998). A synthetic pheromone has been used to help control the false codling moth (Blakey and Wolstenholme, 2014). Biological control using a naturally occurring complex of coccinelids and parasitoid wasps is effective against scale, but only if this complex is protected (De Villiers and van den Berg, 1987).

Notable diseases affecting avocado production include sunblotch caused by the avocado sun blotch viroid (ASBVd); black spot on fruit caused by the fungus *Pseudocercospora purpurea*; anthracnose caused by fungi of the *Colletotrichum* genus; stem end rot caused by several fungi including *Dothiorella aromatica*, *Lasiodiplodia theobromae* and *Colletotrichum gloeosporioides*; stem cankers caused by oomycetes of the *Phytophthora* genus, such as *P. cactorum* and *P. citricola*; and PRR caused by *Pc* (Schaffer *et al.*, 2013, DAFF, 2017a). ASBVd, black spot,

anthracnose and stem end rot cause direct damage to avocado fruit as lesions and discolouration, which reduces sale value, especially for the export market. Phytophthora diseases and ASBVd affect tree growth, which leads to lower fruit yield. As with insect pests, there is an impetus to reduce the use of chemical control for environmental and consumer concerns and to rather use integrated disease management. Copper oxychloride sprays are effective against black spot, anthracnose, and stem end rot pathogens (Darvas and Kotzé, 1987). Other control methods include sanitation practices, roguing, harvesting practices and biological control. Sanitation practices, such as removal of dead fruit, branches and leaves reduce pathogen inoculum and are fairly effective against black spot and anthracnose (Darvas, 1982, Marais, 2004). Roguing and destruction of infected plants is important in controlling ASBVd spread in nurseries and in the field (Schaffer *et al.*, 2013). Harvesting dry fruit is effective in reducing fruit damage by stem end rot (Darvas and Kotzé, 1987) and biological control by the use of *Bacillus subtilis* sprays can be effective against black spot, anthracnose and stem end rot (Korsten *et al.*, 1993, Korsten *et al.*, 1997). Control measures against Phytophthora cankers are similar to PRR, which is discussed in more detail in the following sections, as it is globally the most detrimental disease of avocado (Ploetz, 2003, Wolstenholme, 2010, Blakey and Wolstenholme, 2014).

2.2 Phytophthora Root Rot of Avocado

2.2.1 The Pathogen

Phytophthora cinnamomi Rands causes Phytophthora root rot. Since this oomycete seldom reproduces sexually due to the required presence of both of the mating types A1 and A2, asexual reproduction is more common. Its aseptate hyphae usually persist

saprophytically in soil and it can survive unfavourable conditions by the production of chlamydospores (Figure 2.1). When soil conditions become favourably warm and wet, the pathogen sporulates and releases biflagellate motile zoospores, which swim chemotactically toward feeder roots to infect them. Soil waterlogging increases the volume of water in which zoospores can swim and thus facilitates dispersal and hence increases infection. The spores prefer to move into furrows formed by anticlinal epidermal walls in zones of root elongation where they quickly adhere and form a protective mucus-like coating and then encyst. Within half an hour, they germinate, producing germ tubes that secrete cell wall degrading enzymes and penetrate the epidermis to infect the host (Hardham, 2001, Hardham, 2005, Hardham and Blackman, 2018). Once inside the roots, hyphae grow through the cortex to the vascular tissue, where they can spread to the rest of the root system (Ruiz Gómez *et al.*, 2015, Hardham and Blackman, 2018).

2.2.2 Symptoms

As the pathogen spreads through the root system, the fine roots rot and change colour from pearl to black as they become necrotic and die. The damaged root system absorbs less water and nutrients, which causes canopy decline. The infected tree produces stunted and chlorotic leaves, which finally wilt and then abscise. This is accompanied by a reduction in fruit yield, as shoots begin to wither and die. The decline continues and eventually results in tree death if the rootstock is susceptible to *Pc* (Zentmyer, 1953, Hardham, 2005, Eskalen, 2008, Oßwald *et al.*, 2014).

2.2.3 Integrated Control

As no single approach offers complete control, integrated management of PRR is used in the form of the “Pegg Wheel” (Pegg, 1978, Wolstenholme, 2010) with each of its spokes pertaining to a different management aspect (Figure 2.2). As *Pc* infection originates in the roots, it is vital to ensure adequate drainage and aeration to avoid stressing the flood sensitive avocado and to restrict zoospore dispersal. Orchard sites should be carefully selected for well-drained soil and the absence of impermeable strata. Finer textured soils with 15 % clay or more, should be ridged before planting to enhance drainage (Wager, 1942, Zentmyer, 1953, Pegg, 1978, Wolstenholme, 1979, Wolstenholme, 2010). Irrigation should be scheduled not only to avoid soil waterlogging, but also to mitigate stress from water deficit, especially in hot weather and at vulnerable tree developmental stages, such as flowering and fruiting. (Wager, 1942, Wolstenholme, 1979, Salgado and Cautin, 2008, Wolstenholme, 2010). Mulching is important as it encourages the growth of microbes antagonistic to *Pc* and organic material improves soil structure to increase drainage and increase water and nutrient retention which benefit tree growth (Wolstenholme, 1979, Coffey, 1987, You and Sivasithamparam, 1995, Giblin *et al.*, 2005, Wolstenholme, 2010). It is recommended that irrigation should be applied by microsprinkler to moisten the mulch for the growth of microbes (Salgado and Cautin, 2008, Wolstenholme, 2010). Another management aspect is mineral nutrition, which should be applied to prevent deficiency stress before symptoms arise. This is best done according to the phenological stages (Table 2.2).

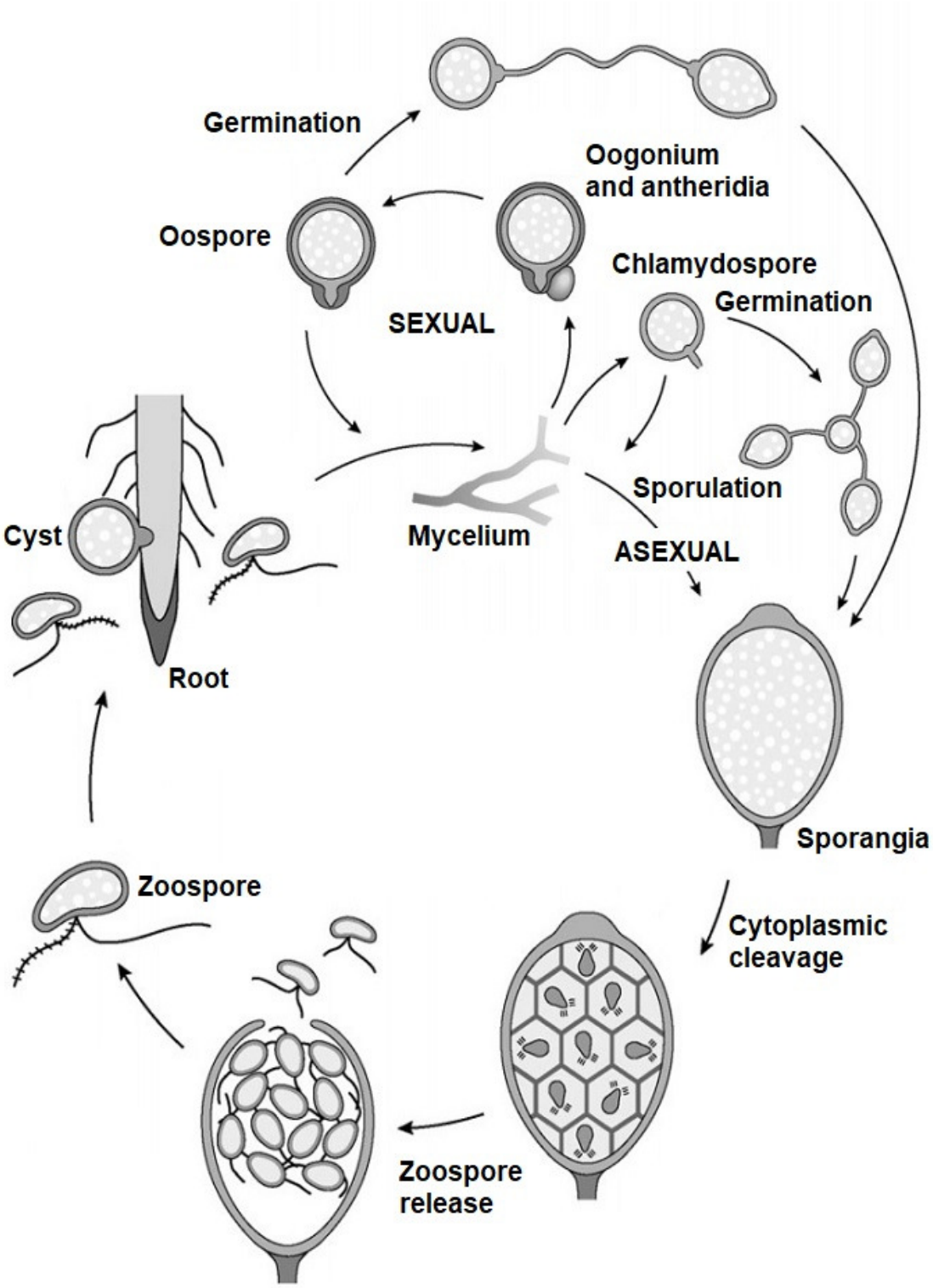

Figure 2.1: The sexual and asexual life cycle of *Phytophthora cinnamomi* (Hardham, 2009).

Table 2.2: Mineral nutrition at each of the phenological stages of avocado (Whiley *et al.*, 1988).

Stage	**Elements**
Around peak flowering and fruiting	P, K, Ca, Zn and B
Around peak vegetative flushes	Zn
At the beginning of the summer root flush	N, K and B
Around the peak of the summer root flush	N, K, B, P and Ca

Nitrogen application is required prior to and during leaf flushes, and is thus used to control the balance between vegetative and reproductive growth. Boron, zinc and potassium applications are necessary as these elements have a tendency to leach below the root zone. Calcium is applied as lime which is used to increase the pH of acid soils, and Ca^{2+} is also a mild fungicide against *Pc* (Wolstenholme, 1979, Whiley *et al.*, 1996, Wolstenholme, 2010). Fungicides are an aspect that can be prudently used to augment control, either by soil application of metalaxyl or by trunk injection, soil and foliar application of phosphonates. These compounds are not only toxic to *Pc*, but phosphonates prime the tree's defence responses for better control of infection (Wolstenholme, 1979, Bezuidenhout *et al.*, 1987, Darvas and Bezuidenhout, 1987, Guest *et al.*, 1995, Wolstenholme, 2010). Lastly, fruiting cultivars should be grafted onto rootstocks resistant to *Pc*. These rootstocks should be grown free of infection in a nursery by using disease-free planting media, and clean irrigation water (Wolstenholme, 1979, Wolstenholme, 2010).

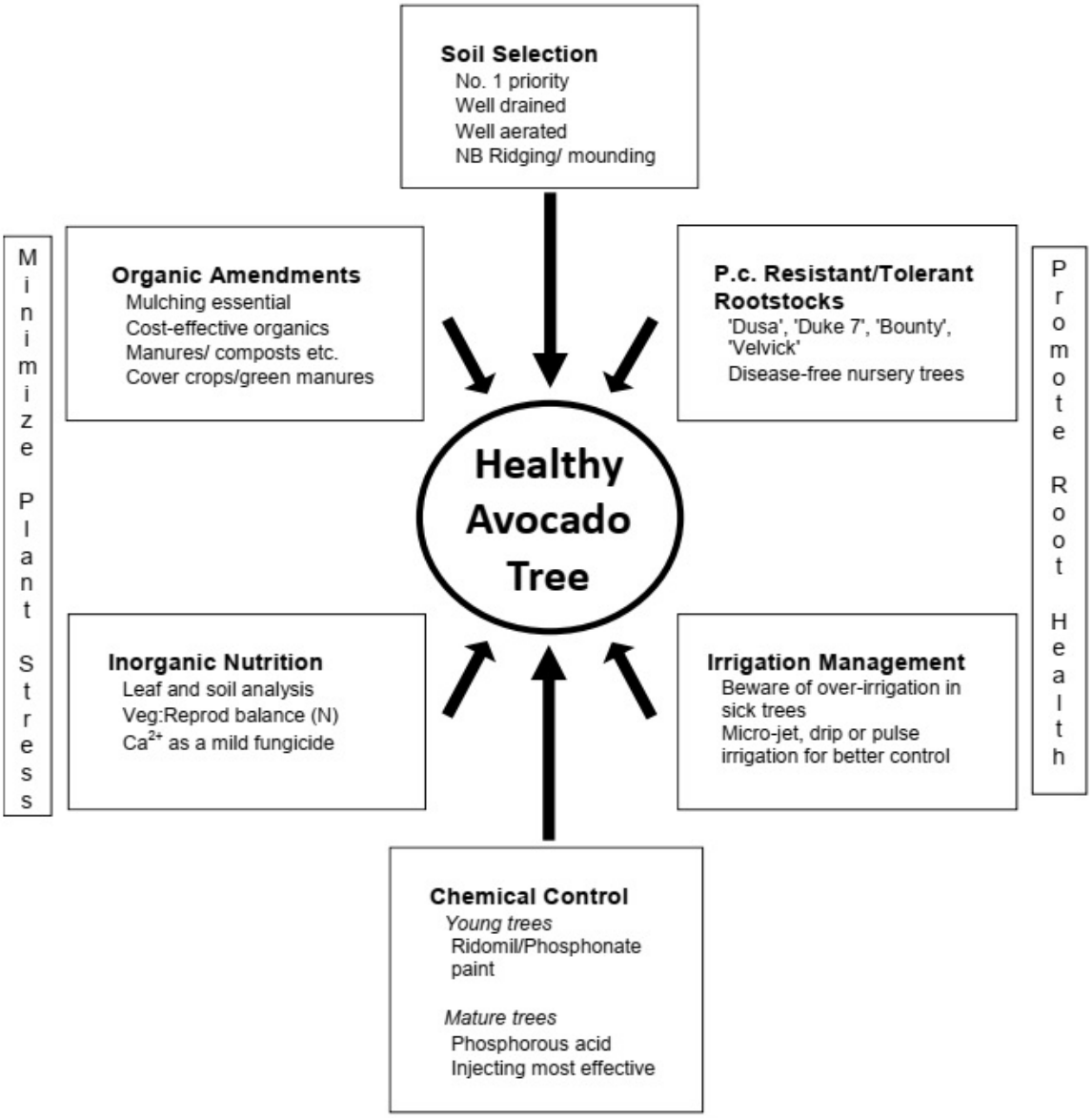

Figure 2.2: The “Pegg-wheel” concept for the integrated control of Phytophthora root rot (Wolstenholme, 2010).

2.2.4 Tolerant or Resistant Rootstocks

Fruit trees typically consist of a rootstock grafted with a mature fruiting scion. This convenient division allows scions to be bred for production, while rootstocks are bred for biotic stress resistance, abiotic stress tolerance and control of tree size. As grafted scions are already mature, they produce fruit much sooner than fruit trees grown from

seed. Scions can also be replaced to suit production and market trends (Hartmann and Kester, 2002). In avocado, rootstocks are primarily bred for tree size reduction which facilitates management, for prevention of alternate bearing which promotes consistency in production, for tolerance to abiotic stresses, which include flooding, soil acidity, high salt and calcium levels and lastly for tolerance and resistance to PRR (Coffey and Guillemet, 1987, Ben-Ya'acov and Michelson, 1995, Wolstenholme, 2003, Schaffer *et al.*, 2013).

Tolerant or resistant rootstocks are bred by allowing the open pollination of resistant or tolerant mother plants which results in a generation of seeds with genetic variation. These seeds are germinated and subsequent plants are infected with *Pc* and grown in a mist bed. Those that show tolerance or resistance to *Pc* are retained as potential rootstocks for further trials and those that show susceptibility or die, are discarded (Violi *et al.*, 2006, Kremer-Köhne and Köhne, 2007). Escape trees, which are chance survivors of unintentional *Pc* infection in the field, are also recruited as potential rootstocks into further trials (Coffey, 1987). For these trials, potential rootstocks are grafted with fruiting cultivars to test their effects on scion growth habit and fruit production. If the resulting effects are favourable, they are passed as tolerant or resistant rootstocks which can be used in commercial avocado production (Bijzet, 1999, Violi *et al.*, 2006, Kremer-Köhne and Köhne, 2007). Tolerant or resistant rootstocks are clonally propagated to prevent variation that would occur during sexual reproduction and possibly result in the loss of tolerant or resistant traits (Ben-Ya'acov and Michelson, 1995). Tolerant and resistant rootstocks currently in use include 'Dusa'™, 'Latas'™ 'Velvick', 'Duke7', and 'Barr Duke' (Ben-Ya'acov and Michelson, 1995, Roe *et al.*, 1998, Wolstenholme, 2003). The aforementioned breeding process,

however, has a high discard rate and takes considerable time and labour (Ben-Ya'acov and Michelson, 1995).

In some cases, the terms resistant and tolerant have been used interchangeably with regard to resistant rootstocks. Resistance is defined as the ability to hinder pathogen growth and infection, whereas tolerance does not hinder pathogen growth and infection, but rather diminishes or counterbalances their negative effects on plant fitness (Horns and Hood, 2012, Pagán and García-Arenal, 2018). The PRR resistant avocado rootstocks R0.06 and R0.09 are termed resistant for the purposes of this dissertation because they have previously shown less pathogen growth compared to susceptible rootstocks in response to infection (Engelbrecht *et al.*, 2013). The mechanism of avocado resistance to PRR is polygenic and hence it is challenging to identify which traits have the highest impact against PRR (Mahomed and van den Berg, 2011). Research into the mechanism of resistance may aid defence marker identification, which could be used to streamline the current laborious breeding and selection process toward increasing resistance. It is believed that the best solution to PRR of avocado would be a highly resistant rootstock (Menge *et al.*, 2002). To that end it is first necessary to mechanistically understand the avocado defence response to *Pc*.

2.3 Plant Defence against Pathogens

Microbes interact with plants in three different ways: mutualism, commensalism and parasitism. In mutualism, both the microbe and the plant benefit; in commensalism only the microbe benefits and in parasitism, the microbe benefits and the plant is harmed. In the last case, the microbe is a pathogen, the plant a host and the result is

plant disease (Agrios, 2005, De Coninck *et al.*, 2015). For pathogenic disease to manifest in a plant, the plant must be a host (susceptible), the microbe a pathogen and the ambient environment suitable. These three requisites for plant disease form the apices of the disease triangle (Francl, 2001, Agrios, 2005). Pathogenicity is the ability of a microbe to cause disease, whereas virulence indicates to what extent it causes disease (Sacristan and Garcia-Arenal, 2008). Organisms such as herbivorous insect pests are not pathogens as they cause biotic stress in plants, but not necessarily disease (Agrios, 2005).

Plant pathogens require hosts in order to gain access to nutrients and to complete their life cycle. There are three pathogen lifestyles that can occur within hosts: biotrophy, necrotrophy and hemibiotrophy (Agrios, 2005, Delaye *et al.*, 2013). Biotrophs have a narrow host range, they dwell covertly in close contact with plant cell membranes or within plant cells and they do not kill the cells to release nutrients. Necrotrophs have a broader host range than biotrophs and they use cell wall degrading enzymes to break down plant cells in order to release nutrients for survival, while killing host cells. Hemibiotrophs have an intermediate to broad host range and they initially exist covertly as biotrophs, but later change strategy and act as necrotrophs (Barras *et al.*, 1994, Agrios, 2005, Glazebrook, 2005, Hardham and Blackman, 2018).

2.3.1 Constitutive and Inducible Defences

To enter into the host, plant pathogens need to pass through sophisticated detection systems and defence mechanisms. Plant defence can be categorised into constitutive and inducible defences, each of which can be further categorised into structural and

chemical defences. Constitutive defences are those continually in place, which include the cuticle, bark, cell walls (with and without secondary thickening) and secondary compounds such as phenolics (Andrews, 1992, Freeman and Beattie, 2008, Vincent *et al.*, 2013). Inducible defences require pathogen detection and activation, and are thought to respond in a manner comprising of two-levels in which the first level involves a general detection and weaker response, but the second level involves a specialised detection and stronger response (Freeman and Beattie, 2008, Eyles *et al.*, 2010).

At the first level of inducible defence, pattern recognition receptors (PRS) housed in the plant cell membrane detect pathogen/ microbial associated molecular patterns (PAMPs/MAMPs) or damage associated molecular patterns (DAMPs). The former (PAMPS/MAMPs) are molecules or regions of molecules that contain motifs unique to pathogens and are usually conserved due to their importance in pathogen metabolism (Jones and Dangl, 2006, Yu *et al.*, 2017). The latter (DAMPs) are products of breakdown and result from a pathogen attacking the plant (Boller and Felix, 2009). The detection causes a signal cascade to the plant cell nucleus to begin transcription of defence genes. This is known as PAMP triggered immunity (PTI) and it is a basal response within hours of infection that does not usually involve programmed cell death (PCD) (Jones and Dangl, 2006, Eyles *et al.*, 2010, Boutrot and Zipfel, 2017, Yu *et al.*, 2017).

At the second level of inducible defence, the pathogen deploys effector molecules that target and disable PTI, leading to effector triggered susceptibility (ETS). Plants can detect pathogen effectors with nucleotide binding site leucine rich repeat receptors (NLRs), which are located in the plant cell membrane and cytoplasm and are coded

for by plant *R* genes (Jones and Dangl, 2006, Kamoun, 2006, Thomma *et al.*, 2011). If the receptors bind to pathogen effectors, a signal cascade is initiated which results in the binding of transcription factors and transcription of plant defence related genes. This results in effector triggered immunity (ETI), which is a response within 2 days of infection that involves the hypersensitive response (HR) and PCD. If the plant fails to detect effectors at this stage it will remain susceptible, become diseased and may die (Jones and Dangl, 2006). Examples of inducible defences include the production of reactive oxygen species (ROS), plant hormones, pathogenesis related (PR) proteins, phytoalexins, HR, stomatal closure and cell wall fortifications by lignin, callose and hydroxyproline rich glycoproteins (Jones and Dangl, 2006, Yu *et al.*, 2017).

The second level of inducible defence is referred to as the “gene for gene” hypothesis, which was proposed by Flor (1971), where *Avr* genes code for effectors in pathogens and *R* genes code for resistance proteins in plants. Later *Avr* proteins were renamed as virulence factors, because they can promote virulence, depending on the host (Chisholm *et al.*, 2006, Jones and Dangl, 2006). Virulence factors have subsequently been renamed effectors, as a single effector may cause virulence or avirulence in different host plant species (Staskawicz *et al.*, 1995, Boller and Felix, 2009).

As plants and pathogens coevolve, plants develop more sophisticated defences against pathogens, and their pathogens must develop even more sophisticated means to infect them (Whitham *et al.*, 2006). This is referred to as the pathogen-host “arms race” (Boller and He, 2009) for which Jones and Dangl (2006) proposed the “zigzag” model to explain the concept. Plants evolve NLRs that successfully detect pathogen effectors and pathogens evolve new effectors to reinfect their plant hosts. This results

in a “zigzag” interplay between effector triggered susceptibility (ETS) and effector triggered immunity (Figure 2.3). Recently, Keller *et al.* (2016) suggested that the “zigzag” model is too inflexible because some necrotrophs promote cell death involved in ETI to provide dead tissue for them to feed. Therefore, the separation between PTI and ETI still remains somewhat indistinct and in future, the “zigzag” model could require refinement (Thomma *et al.*, 2011).

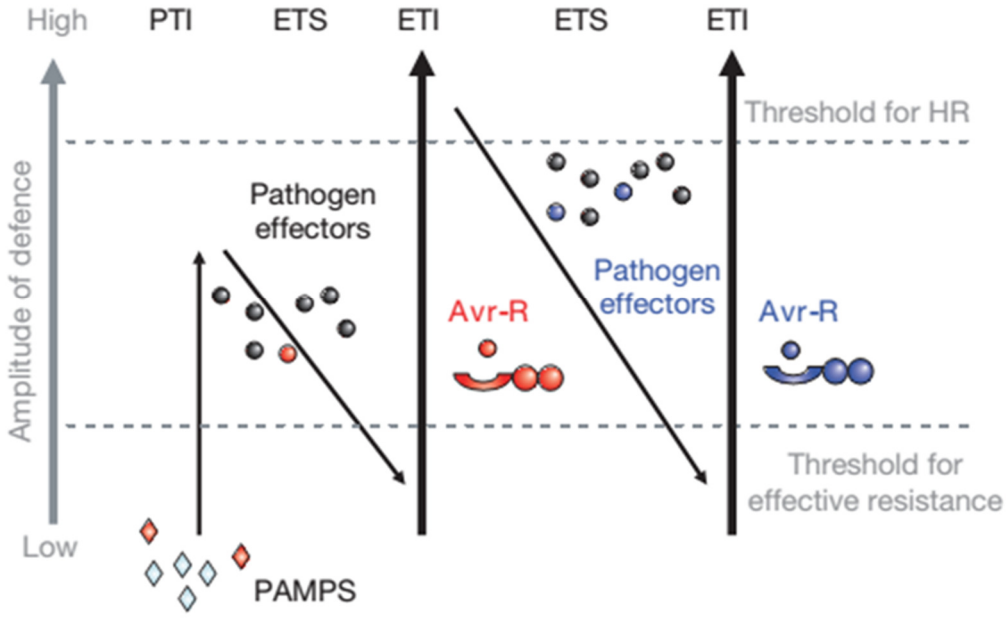

Figure 2.3: The “zigzag” model of plant defence (Jones and Dangl, 2006). Plants detect pathogen associated molecular patterns (PAMPS), leading to pathogen triggered immunity (PTI). Pathogens secrete effectors to bypass pathogen triggered immunity, leading to effector triggered susceptibility (ETS). Effectors are then detected by evolved receptors (Avr-R) leading to effector triggered immunity (ETI). Pathogens then secrete evolved effectors, triggering susceptibility and the zigzag process continues.

Successful pathogen detection, system wide signalling and defence results in systemic acquired resistance (SAR) of a plant to a pathogen. Failure of a plant to halt infection by plant defence systems will result in disease and possible death to the plant (Ryals *et al.*, 1996, Durrant and Dong, 2004). To help solve PRR of avocado, several common inducible defence responses that are prominent among plants will be investigated in this study, namely callose, lignin, phenolics, ROS, antioxidants, tyloses and gum. These are explained in due course.

2.3.2 Infection Strategy of *P. cinnamomi*

Upon reaching the root surface, zoospores release their flagella, secrete mucilage and adhesins and then encyst. The encysted spore germinates and grows a germ tube between anticlinal cell walls, which secretes a cascade of different cell wall degrading enzymes, and can form an appressorium like structure to force its entry as it begins penetration through the plant cell wall. Having breached the cell wall, the pathogen forms haustoria against the plant cell membrane to absorb nutrients and secrete effectors in the biotrophic phase, thereafter, the pathogen becomes necrotrophic and feeds off dead tissue (Hardham, 2001, Hardham, 2005, Hardham and Blackman, 2010, Hardham and Blackman, 2018).

The first constitutive defence the pathogen encounters is the plant cell wall, but the plant root cell needs to detect the PAMPS, or effectors, before the cell wall is breached for the successful initiation of induced defences. These induced defences include a burst of signal ROS and secondary cell wall thickening by callose papillae and by lignin from phenolic monomers in the presence of ROS (Ellinger and Voigt, 2014, Hückelhoven, 2014, Barros *et al.*, 2015, Bigeard *et al.*, 2015). If the pathogen breaches

the cell wall undetected, the plant cell has another opportunity to detect effectors secreted by haustoria and undergo HR to block the spread of the pathogen. If detection is successful at this stage, a burst of ROS will be generated and the cell will be sealed with lignin, causing cell death during HR (Pontier *et al.*, 1998, Menden *et al.*, 2007). However, if at this point the pathogen changes mode from biotrophy to necrotrophy and begins to feed off of the dead cell, it is questionable if HR will successfully stop the progression of the infection. Furthermore, the effectiveness of lignin in resistance during this interaction is still uncertain. If the pathogen does manage to reach the stele and penetrate the xylem and if the plant manages to produce tyloses and gum, this could potentially halt the pathogen spread to other parts of the root system (Phillips *et al.*, 1987, De Micco *et al.*, 2016).

2.3.3 Callose

Callose is a helical biopolymer of β-glucose found in plants that is synthesised by callose synthase enzymes and can be degraded by plant or pathogen glucanases (Bacic *et al.*, 2009). Its helical structure and ability to be enzymatically deposited and removed, makes it versatile and multifunctional in plants (Galatis and Apostolakos, 2010). Some of its functions include control of transport through plasmodesmata, importance in pollen development and pollen tube growth, cell plate and wall formation and deposition in response to abiotic and biotic stresses (Chen and Kim, 2009, Piršelová and Matušíková, 2013, Ellinger and Voigt, 2014). In order to understand its function in response to biotic stress, it is first necessary to understand the structure of callose, beginning with glucose.

Glucose is a hexose monosaccharide with a six-carbon backbone. Within a linear glucose molecule, the first carbon in the backbone forms part of an aldehyde group, the next four carbons are each bonded to a hydrogen and a hydroxyl group and the last carbon is bonded to two hydrogens and a hydroxyl group. As carbon has a valency of four, glucose exists in both L and D optical isomer forms and plants naturally produce the D-isomer (Figure 2.4). The aldehyde group of carbon one and the hydroxyl group of carbon five can bond together to produce a hemiacetal ring form of glucose called a pyranose (Figure 2.5). As a result of this bond, the carbon one can have its hydrogen and hydroxyl group oriented in two different conformations: the hydroxyl group below the ring in α-glucose and above the ring in β-glucose. The latter is the conformation which is found in callose (Bacic *et al.*, 2009, Campbell, 2009).

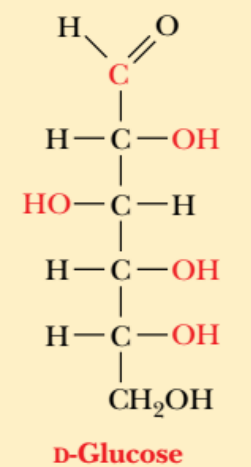

Figure 2.4: D-glucose (Campbell, 2009). The aldehyde group is on the first carbon at the top, followed by the next four carbons with hydrogens and hydroxyl groups and lastly the sixth carbon is shown with two hydrogens and a hydroxyl group attached.

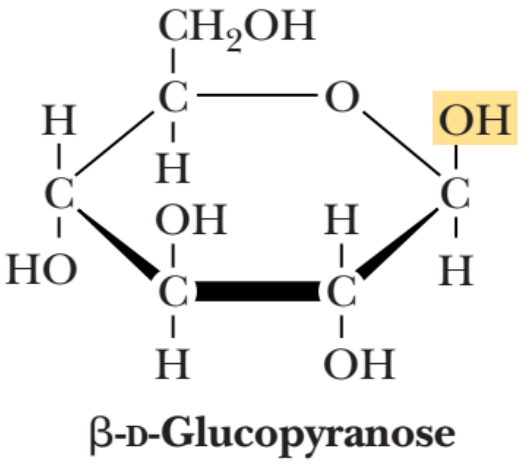

Figure 2.5: β-glucose (Campbell, 2009). The pyranose form is shown with carbons one and five having formed a hemiacetal group containing the oxygen in the ring. The hydroxyl group above the ring on carbon one is highlighted showing the β conformation.

The existence of different ring conformations and presence of many hydroxyl groups allows for condensation reactions and complex glucose polymer formation in the presence of relevant enzymes (Campbell, 2009, Book, 2014). The elimination of a water molecule by an enzyme from two bonding hydroxyl groups results in glycosidic bond formation. The type of glycosidic bond determines the 3-D conformation of the polymer and thus its biological function. The callose synthase (Figure 2.6) used in defence is GSL-5 and forms the 1-3 glycosidic bonds in callose, which have a bent bond angle. This angle results in callose being a coiled or helical molecule, well suited to bolstering, scaffolding or sealing (Galatis and Apostolakos, 2010), and can therefore be used to bolster cell walls against pathogen entry (Jacobs *et al.*, 2003, Piršelová and Matušíková, 2013, Ellinger and Voigt, 2014). As a pathogen begins to penetrate the cellulose cell wall, callose synthase enzymes are relocated to these sites in the cell membrane to produce callose, which then integrates with the cellulose cell wall

and bolsters it against penetration, forming a plug called a papilla (Figure 2.7) (Eggert *et al.*, 2014).

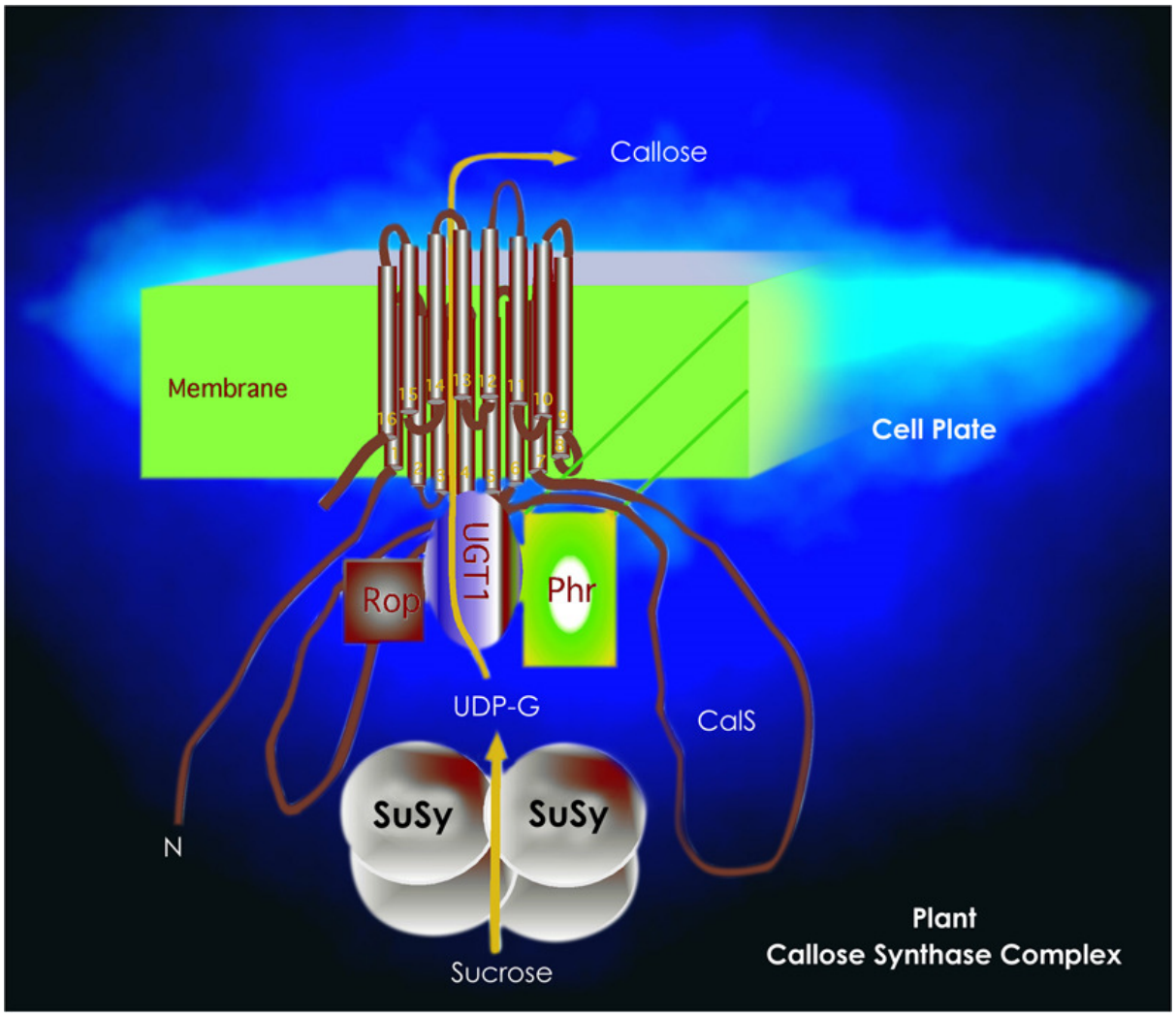

Figure 2.6: Model of Arabidopsis callose synthesis at the cell plate (Hong and Verma, 2007). Rop is a Rho-like GTPase, Phr is phragmoplastin, UGT1 is UDP-glucose transferase, UDP-G is UDP-glucose and SuSy is sucrose synthase. UGT1 interacts with Phr and Rop as sucrose is converted by SuSy to UDP-G, which is then used as the substrate to produce callose from callose synthase (CalS).

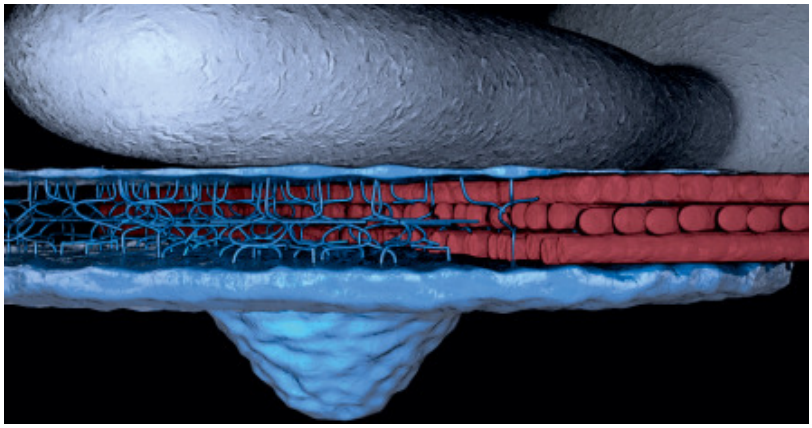

Figure 2.7: A model papilla produced in response to fungal infection (Eggert *et al.*, 2014). The papilla is in blue, the cellulose cell wall in red and the fungal structures in grey.

Resistant plants that have produced callose in response to infection include a resistant line of *Arabidopsis thaliana* in response to *Golovinomyces cichoracearum* (Eggert *et al.,* 2014) and *Glycine max* in response to *P. sojae* (Enkerli *et al.,* 1997). In *Pc*-resistant species, early callose deposition is often observed in papillae as an infection response, but this callose deposition is often absent in susceptible species. Some examples include resistant *Gahnia radula*, *Acacia pulchella*, *Zea mays* and *Triticum aestivum*, which produced callose in response to infection, whereas susceptible *Lupinus angustifolius* and *Eucalyptus marginata* did not produce callose (Hinch and Clarke, 1982, Cahill and Weste, 1983, Allardyce, 2011). Furthermore, early callose deposition has been observed at 6 hours after infection in a resistant R0.06 avocado rootstock, whereas a susceptible R0.12 rootstock only produced callose after 96 hours (van den Berg *et al.*, 2018a). Early callose deposition in response to infection is therefore potentially valuable in avocado defence studies, and may aid in the identification of resistance markers.

2.3.4 Reactive Oxygen Species

Reactive oxygen species are chemical species containing oxygen that are formed from oxygen by energy or electron transfer (Gill and Tuteja, 2010), which makes them reactive. Examples include the superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), the peroxide ion (O_2^{2-}), the hydroxide radical ($OH^{\cdot}$) and the hydroxyl ion (OH^-) (Held, 2010). ROS species have different half-lives and reactivities, and those that are more reactive tend to have a shorter half-life. For example $OH^{\cdot}$ is highly reactive and only has a half-life of approximately 10^{-9} seconds, whereas H_2O_2 is less reactive and has a half-life of 10^{-3} seconds (Sies, 1993, D'autréaux and Toledano, 2007). The more reactive the ROS species, the more damage it can cause. At high concentrations, ROS are known to cause significant damage to macromolecules such as DNA, lipids and proteins, and at low concentrations ROS are produced for systemic signalling (Apel and Hirt, 2004, Mittler *et al.*, 2004, Gill and Tuteja, 2010, Baxter *et al.*, 2014).

Plants need to control ROS production, whether it be to moderate oxidative damage or to fine-tune signalling, and hence they produce antioxidants, which are classed as either enzymatic or non-enzymatic (Blokhina *et al.*, 2003). Within the class of enzymatic antioxidants, there are several common families, and within them, there is variation in their structure, cellular location and the specific cycle in which they function. However, each family catalyses the same type of reducing reaction. Family examples include peroxidases, reductases and dismutases (Mittler, 2002, Mittler *et al.*, 2004, Gill and Tuteja, 2010). Non-enzymatic antioxidants include a variety of molecules such as ascorbate, tocopherols, tocotrienols, carotenoids and phenolics. Ascorbate works in certain cycles in cooperation with enzymatic antioxidants, whereas tocopherols and carotenoids work to manage oxidative stress in membranes (Blokhina

et al., 2003, Apel and Hirt, 2004, Gill and Tuteja, 2010). Together, all these antioxidants moderate and fine-control ROS production in cells.

ROS are normally produced in plants as products and by-products of plant metabolism, in responses to biotic and abiotic stress and in systemic stress signalling (Mittler, 2002, Mittler, 2006, Baxter *et al.*, 2014, Gilroy *et al.*, 2014). Production of ROS from metabolism and biotic and abiotic stresses are largely found in chloroplasts during photosynthesis, mitochondria during respiration and in various reactions in peroxisomes. These organelles are all characterised by a high electron flux in oxidising conditions, which is favourable for ROS production (Mittler *et al.*, 2004).

ROS are produced for the purpose of systemic stress signalling at lower concentrations by cell membrane-bound enzymes and released into the apoplast. Due to its connectivity, the apoplast is used as a ROS signalling corridor to transmit signals to distal plant parts, so that plant-wide response to the stress can be initiated for the objective of plant survival (Gilroy *et al.*, 2014). The plant enzyme family responsible for ROS production is the respiratory burst oxidase homologs (RBOHs), and RBOHD, in particular, is associated with defence signalling. This enzyme contains heme and uses electrons from cellular NADPH passed through flavine adenine dinucleotide (FAD) to generate and release superoxide into the apoplast (Figure 2.8) (Suzuki *et al.*, 2011, Gilroy *et al.*, 2014). The enzyme can be triggered to produce superoxide by the calcium sensitive EF hand domains on the cellular side of the enzyme. The superoxide produced is converted by apoplastic superoxide dismutase (SOD) into the more stable peroxide for further signal transduction (Sies, 1993, D'autréaux and Toledano, 2007, Suzuki *et al.*, 2011). As the ROS signal travels radially outward in the apoplast from

its origin, it is detected by adjacent cells, which trigger a calcium flux from their apoplast and vacuoles into their cytoplasm. Calcium can move from vacuoles to cytoplasm through a pore protein (TPC1). This calcium can propagate as a signal from cell to cell through plasmodesmata, and through the help of a calcium dependent kinase (CPK5/26), can trigger RBOHD to amplify and propagate the ROS signal by generating and releasing more ROS into the apoplast, which occurs radially outward from the signal origin (Figure 2.9) (Suzuki *et al.*, 2012, Gilroy *et al.*, 2014). This signal is known as the oxidative burst and can travel at a speed of 14 mm per second (Gilroy *et al.*, 2014). The burst can be single, or can be biphasic as a double spike in ROS concentration over time (Baxter *et al.*, 2014). The first spike can be triggered by wounding or the detection of pathogens, but the second spike can only be triggered by the detection of pathogen effectors, such as those produced by *Pc* (Stael *et al.*, 2015, Hardham and Blackman, 2018).

The production of ROS has been associated with resistance in an *A.thaliana* line infected with *Pseudomonas syringae*, where Chaouch *et al.* (2012) showed that ROS was produced by the AtRbohF (*A.thaliana* RBOH F). Microscopy studies on *Pc* infected plants linked peroxide produciton with resistance in *Z. mays* roots (Allardyce *et al.*, 2013), and tolerance in an *A. thaliana* line (Rookes *et al.*, 2008). García-Pineda *et al.* (2010) studied avocado roots infected with *Pc* and observed that ROS were produced at 72 hours and 96 hours after infection. However, van den Berg *et al.*, (2018b) observed the early upregulation of transcripts associated with ROS production in *Pc* infected resistant avocado rootstocks. Therefore, the early production of ROS in response to infection may be valuable in avocado defence studies, and may help in the identification of resistance markers.

As early production of ROS may be associated with resistance in avocado, the subsequent production of antioxidants may be required to attenuate ROS after it has performed its defence function. The following studies that involved antioxidants are noteworthy. Acosta-Muñiz *et al.* (2012) found an increase in the expression of proteins associated with antioxidant production at 24 hpi in *Pc* infected tolerant 'Martin Grande' rootstocks, and van den Berg *et al.* (2018b) found the increased expression of transcripts associated with antioxidant production in *Pc* infected resistant 'Dusa'™ rootstocks. Furthermore van den Berg *et al.* (2018a) found that resistant R0.06 rootstocks showed earlier increased SOD activity and increased catalase activity as compared to susceptible R0.12 rootstocks after *Pc* infection. These studies suggest that antioxidants may be associated with resistance and could help to investigate the role of ROS in avocado defence studies with *Pc*.

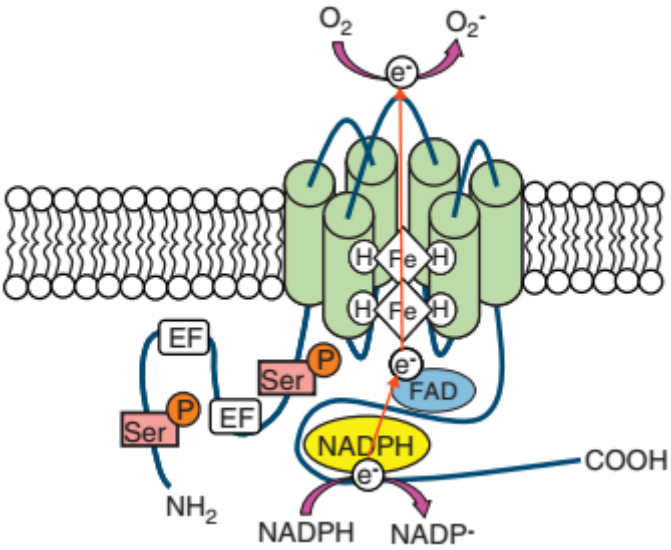

Figure 2.8: The production of superoxide by a respiratory burst oxidase homolog (RBOH) enzyme (Suzuki *et al.*, 2011). The enzyme in green, is housed in the cell membrane. The electron carriers reduced nicotinamide adenine dinucleotide (NADPH) and flavine adenine dinucleotide (FAD) are shown in yellow and blue respectively, in association with electrons (e^-). Heme contains Fe. The enzyme protein begins with NH_2 and ends with COOH. It contains EF hand domains and serine residues (Ser).

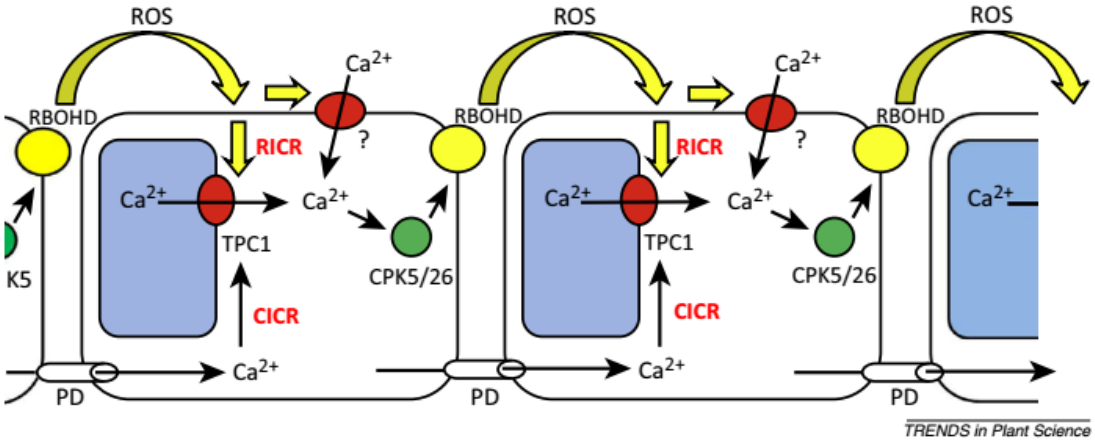

Figure 2.9: Systemic stress signal transmission through adjacent plant cells (Gilroy *et al.*, 2014). The enzyme respiratory burst oxidase homolog D (RBOHD) is shown in yellow, the movement of reactive oxygen species (ROS) by yellow arrows, the movement of calcium by black arrows, pore proteins are shown in red, calcium dependent kinase (CPK5/26), in green, plasmodesmata (PD) in white and vacuoles are shown in blue. Release of calcium is by calcium induced ROS release (CICR) or ROS induced calcium release (RICR).

2.3.5 Phenolic Compounds

Phenolic compounds contain the phenol functional group, which is composed of a hydroxyl group joined to an aromatic hydrocarbon group (Taiz and Zeiger, 2010). In plants, phenolics are a large and diverse family of secondary compounds with multiple functions, which include moderating oxidative stress, structural support, pigmentation, flavouring, and defence against pathogens and herbivores (Taiz and Zeiger, 2010). Most phenolics are produced through the phenylpropanoid pathway, where phenylalanine, produced by the shikimic acid pathway, and malonyl-CoA, from the malonic acid pathway, are utilised (Figure 2.10) (Hahlbrock and Scheel, 1989, Taiz and Zeiger, 2010, Vogt, 2010). Important phenolics involved in defence include

salicylic acid used for signalling; tannins, which bind to proteins to prevent digestion in herbivores; coumarins, which are anti-microbial compounds and cell wall bound phenolics and lignin, which reinforce cell walls against pathogen entry (Dixon and Paiva, 1995, Bhattacharya *et al.*, 2010, Cheynier *et al.*, 2013).

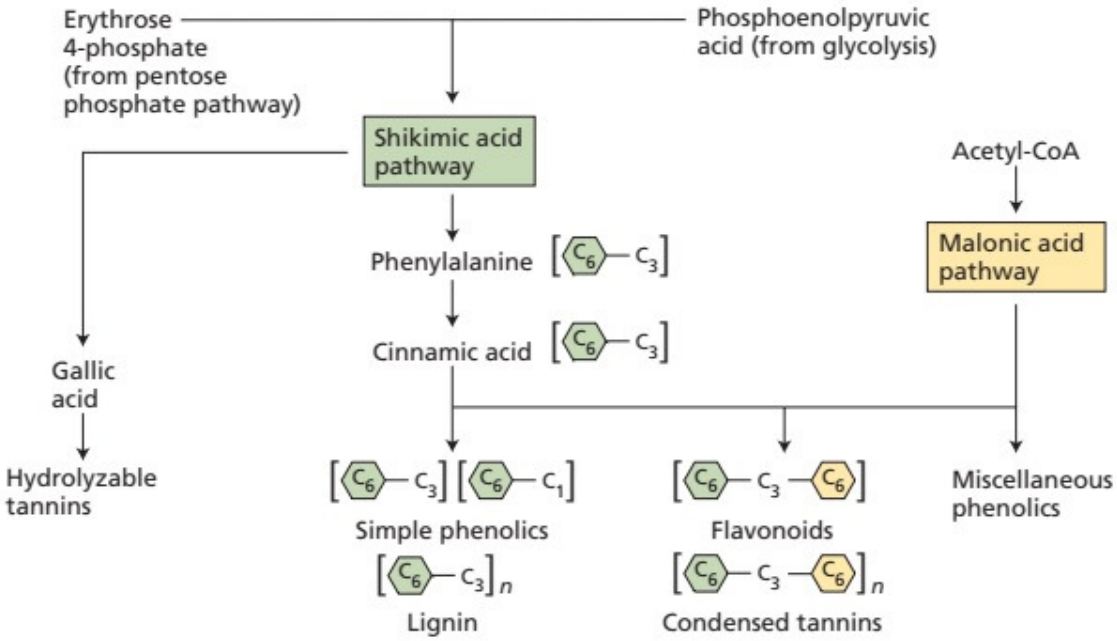

Figure 2.10: Phenolic biosynthesis in plants (Taiz and Zeiger, 2010). The phenylpropanoid pathway begins with phenylalanine. In some cases this pathway uses malonyl-CoA from the malonic acid pathway to form condensed tannins and flavonoids. Hydrolysable tannins and salicylic acid are formed from products of the shikimic acid pathway.

The accumulation of phenolics in the cytoplasm is of interest to this study, because they can be used for any number of defence functions. In studies on *Pc* infected plants, Cahill *et al.* (1993) found that resistant lines of micropropagated *E. marginata* showed higher phenylalanine ammonia lyase (PAL) activity and increased phenolic production.

In a later study, Cahill *et al.* (1989) observed that the production of phenolics and tannins were more likely to be observed in *Pc*-resistant rather than susceptible species. In a study concerning avocado rootstocks infected with *Pc*, van den Berg *et al.* (2018a) found an association of phenolics with susceptibility as a susceptible R0.12 rootstock produced more phenolics as compared to a resistant R0.06. In contrast to this, other studies have found the production of phenolics to be associated with resistance. Namely, Engelbrecht and van den Berg (2013) observed an early downregulation of PAL transcripts in R0.12 and the moderately field resistant 'Duke7' rootstocks but an upregulation in resistant 'Dusa'™ rootstocks. Furthermore, van den Berg *et al.* (2018b) observed gene expression involved with the phenolic class of chalcones in 'Dusa'™. Lastly, Phillips *et al.* (1987) observed tannins and phenolics at 24 and 48 hours post inoculation (hpi) in *Pc* infected 'Duke 7'. Hence phenolic production in avocado rootstocks may be associated with resistance to *Pc*.

2.3.6 Lignin

Lignin, which is named after the Latin word for wood, is a phenolic biopolymer found in plants. It is deposited between cellulose microfibrils to reinforce, support and/or to waterproof cell walls (Barros *et al.*, 2015). Examples include support in sclereid cells; support and waterproofing in xylem tracheids; support in response to mechanical stress or wounding; and also protection and waterproofing during encounters with pathogens (Higuchi, 1990, Tyree and Zimmermann, 2002, Moura *et al.*, 2010, Cabane *et al.*, 2012, Trupiano *et al.*, 2012). The function of lignin in plant defence is to reinforce the cellulose cell wall to prevent pathogen entry and protect the cell, or to seal off the cell during the HR, which causes cell death but prevents further pathogen spread (Moerschbacher *et al.*, 1990, Nicholson and Hammerschmidt, 1992).

Lignin is produced from three main monomers, namely the hydroxyphenyl (H-unit), guaicyl (G-unit) and syringyl (S-unit) monolignols (Barros *et al.*, 2015). These monolignols are synthesised by the phenylpropanoid pathway in the cell protoplast and are then transported by three mechanisms across the cell membrane into the apoplast for polymerisation. The three transport mechanisms are diffusion through plasmodesmata, exocytosis and active transport via a membrane transporter which uses adenosine triphosphate (ATP) (Boerjan *et al.*, 2003, Barros *et al.*, 2015). To aid polymerisation in the apoplast, an RBOH enzyme in the cell membrane uses NADPH to produce superoxide. The superoxide is converted by SOD into oxygen and peroxide which are then used by apoplastic enzymes, (such as laccases and peroxidases), to convert apoplastic monolignols into radicals (Barros *et al.*, 2015). With the help of dirigent proteins (DiP), these radicals oxidatively cross link and polymerise into the polymer lignin (Figure 2.11) (Humphreys and Chapple, 2002, Boerjan *et al.*, 2003, Barros *et al.*, 2015). The composition of lignin varies widely and depends on function, cell type, the ratio of monomer types produced and stereochemistry (Terashima and Fukushima, 1988, Ralph *et al.*, 2008). The extent of lignin production also varies from only primary cell wall thickening to primary and secondary cell wall thickening (Figure 2.12). For example, xylem tracheids show lignin thickening in both primary and secondary cell walls and tends to be composed mainly of G-units, whereas cells responding to biotic and abiotic stress show only primary cell wall thickening, with lignin composed of H- and G-units (Higuchi, 1990, Cabane *et al.*, 2012, Barros *et al.*, 2015).

Lignin deposition has proved important to resistance in lines of *Solanum lycopersicum* and *Gossypium hirsutum* infected with *Verticillium dahliae* (Gayoso *et al.*, 2010, Xu *et*

al., 2011). In studies involving *Pc*, the role of lignin in defence has been variable. Cahill and M^{c}Comb (1992) observed increased lignin deposition in field resistant lines of *E. calophylla* as compared to susceptible lines of *E.marginata*. Cahill *et al.* (1989) observed lignin deposition in *E. calophylla*, *E. maculata*, *A. pulchella*, *G. radula*, *Z. mays* and *T. aestivum*, which appeared to halt infection whereas lignin deposition in *Themeda australis* was bypassed by *Pc* hyphae. In studies involving avocado, the role of lignin in defence has also varied. Infected *Pc*-resistant 'Dusa'™ rootstocks showed the upregulation of genes coding for proteins involved with lignin biosynthesis (Reeksting *et al.*, 2014b, van den Berg *et al.*, 2018b). Furthermore Acosta-Muñiz *et al.* 2012 observed the increased expression of proteins related to lignin biosynthesis in *Pc* infected tolerant 'Martin Grande' rootstocks. García-Pineda *et al.* (2010) found no changes in lignin production in a *Pc* infected susceptible rootstock. These studies suggested that lignin deposition is possibly linked to *Pc*-resistance in avocado, however, van den Berg *et al.* (2018a) observed lignin deposition in a susceptible R0.12 rootstock but not in a resistant R0.06, which suggested lignin was associated with susceptibility. Further investigation of early lignin deposition in response to *Pc* infection in avocado rootstocks is therefore required to reveal its relevance to defence.

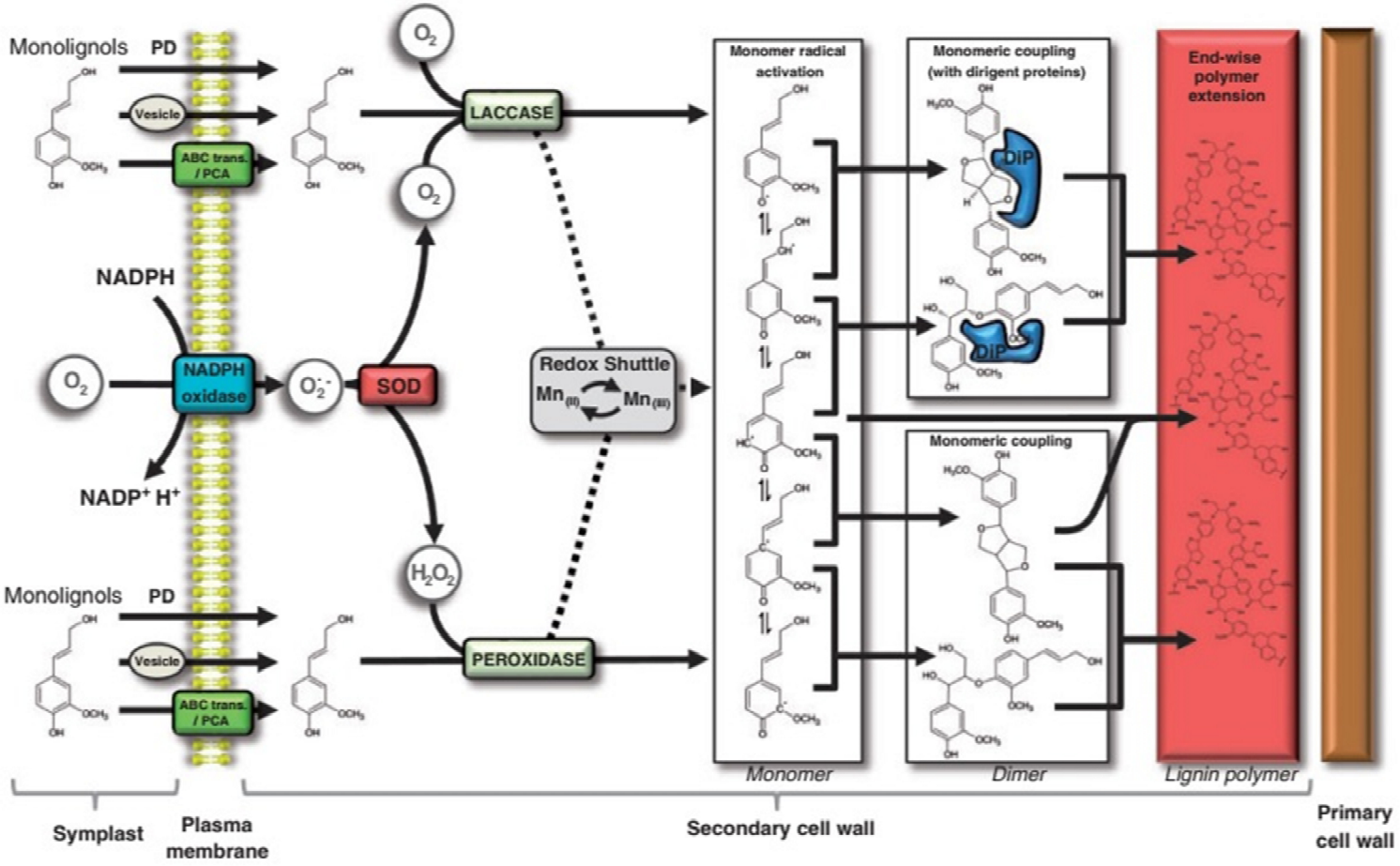

Figure 2.11: Lignin biosynthesis (Barros *et al.*, 2015). Transport of monolignols into the apoplast is shown top and bottom. Protein transporters are shown in green and plasmodesmata as PD. The respiratory burst oxidase homolog (RBOH) is shown in blue and superoxide dismutase (SOD) in red. The redox shuttle is associated with peroxidase and laccase. Dirigent proteins are shown as DiP in blue.

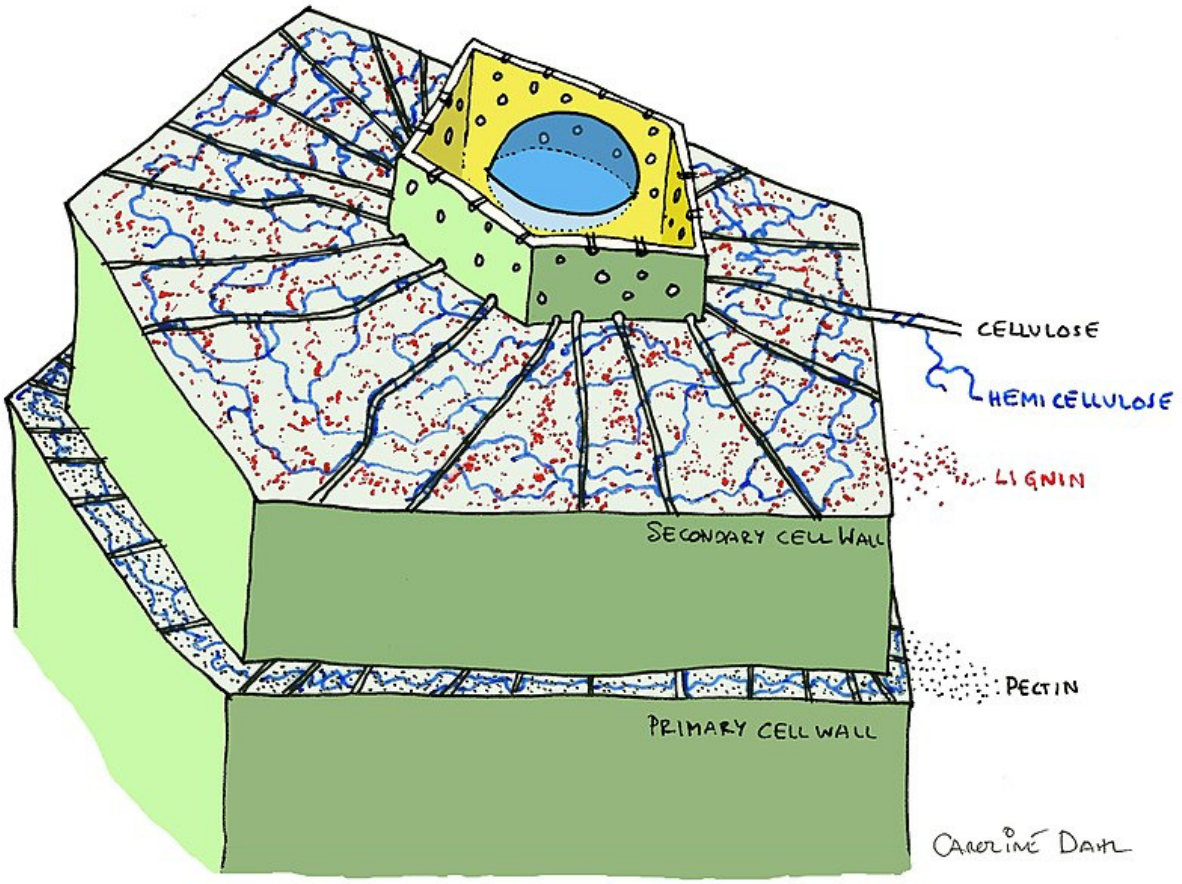

Figure 2.12: Primary and secondary plant cell walls (Dahl, 2011). This diagram shows the primary and secondary cell wall and associated polymers.

2.3.7 Tyloses and Gums

Tyloses and gum are produced in plants to block xylem vessels and to stop sap flow. They are produced normally as trees age but are also produced in response to abiotic and biotic stress (De Micco *et al.*, 2016). Tyloses (singular: tylosis), which are named after the Greek word for sack, are parenchyma cells that grow into xylem vessels through pitted walls (Zimmermann, 1979). Once they occlude the vessel lumen, tyloses undergo secondary thickening with lignin and suberin, which makes them impermeable and thus able to block sap flow (Parke *et al.*, 2009). Tyloses are associated with gums which are polysaccharide and pectin based substances, and

are secreted into xylem vessels from adjacent cells (Rioux *et al.*, 1998). Gel formation is thought to precede gum formation (Agrios, 2005). During pathogen entry into vascular tissue, the blocking of xylem vessels with tyloses and gums prevents the further spread of the pathogen (De Micco *et al.*, 2016).

In an avocado cultivar susceptible to *Raffaelea lauricola*, Inch *et al.* (2012) observed the formation of tyloses and gels at 14 days post inoculation. Two microscopy studies done on avocado rootstocks infected with *Pc*, have observed the formation of tyloses. Phillips *et al.* (1987) observed tyloses in moderately field resistant 'Duke 7'. Van den Berg *et al.* (2018a) observed tyloses in a susceptible R0.12 at 3 hours after infection, which was earlier than those observed in a resistant R0.06 at 12 hours after infection. Although defences closer to the root epidermis are more important to prevent the early spread of the pathogen, the role of tyloses and gums may be of importance to avocado resistance to *Pc*, but require investigation.

2.4 Conclusion

This review has explored the literature concerning the early responses of avocado rootstocks to infection with *Pc* and the associated concepts. This has provided a foundation for further experimental work in subsequent experimental chapters in the dissertation. It is notable that information on the response of avocado rootstocks to *Pc* infection is still lacking, particularly: the production of callose, tyloses and gum in R0.09 and R0.38; and also the production of ROS, total antioxidants and lignin in R0.09, R0.38 and R0.06. The investigation of these responses to infection could aid with defence marker identification and ultimately assist in solving the Phytophthora root rot of avocado conundrum for the benefit of the avocado industry.

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Chapter 3

Histochemical differences in the response of Avocado rootstocks to infection with *Phytophthora cinnamomi*

3.1 Introduction

The status of avocado as a global fruit tree crop and the loss in production due to Phytophthora root rot (PRR), has already been highlighted in the previous chapter. As an aspect of integrated disease management of PRR, the use of tolerant and resistant rootstocks is important, but the breeding and selection of these rootstocks requires much time and effort (Kremer-Köhne and Köhne, 2007, Wolstenholme, 2010). Marker assisted selection may help to streamline the rootstock selection process, however, it requires prior identification of defence markers. This identification could be assisted by investigating the difference in the defence response between resistant and susceptible avocado rootstocks infected with *Phytophthora cinnamomi* (*Pc*). In the qualitative investigation of these differences, confocal microscopy is a useful tool as it provides the means and the resolution to examine cellular responses visually, as opposed to chemically or quantitatively. This advantageously allows for the location of responses in different parts of the root, but due to the thin sectioning of tissue, this is limited to a relatively small volume of the total root tissue. The following defence responses were investigated by microscopy: callose and lignin deposition, synthesis of phenolic compounds and the production of tyloses and gum in xylem vessels. The hypothesis for this chapter was: earlier or increased production of callose and phenolics are associated with resistance to *Pc* and an earlier or increased production of lignin and tyloses and gum are associated with susceptibility to *Pc*. The main aim of the chapter was to compare the early infection response of resistant and susceptible rootstocks and in order to test the hypothesis, the objective was to observe the location and relative amount/number of responses in the first 24 hours post inoculation (hpi).

3.2 Methods and Materials

Filters for different ranges of light wavelengths were used to observe different cellular responses and these observations were combined into a single image for analysis. Callose deposition was examined using a calcofluor stain and a blue wavelength filterset whereas lignin deposition, phenolic accumulation and presence of tyloses and gums were examined using autofluorescence and a red wavelength filter set (Phillips *et al.*, 1987, van den Berg *et al.*, 2018). Calcofluor is a fluorophore that stains both chitin and beta glucans such as callose and cellulose, but can be used to successfully observe callose production in response to *Pc* infection (Hughes and M^{c}Cully, 1975, Herth and Schnepf, 1980, Wood *et al.*, 1983, van den Berg *et al.*, 2018).

3.2.1 Plant Material

The clonal avocado plants for this study were provided by Westfalia Technological Services in Tzaneen, Limpopo Province, South Africa and consisted of two resistant (R0.09 and R0.06) and one susceptible (R0.38) rootstock. The plants were transplanted into 5 L black nursery bags, which contained a 50:50 vermiculite and perlite mix, and were then placed under a shade net structure on the Hatfield Experimental Farm at the University of Pretoria (25° 45' 06.6" S 28° 15' 47.1" E). Over the next three months, plants were fertigated via arrow drippers, three times a day for 15 mins at a time, with a daily total of 1.5 L of 1 g/L Hygroponic and 0.7 g/L Solu-Cal fertiliser solution (Hygrotech SA (Pty) Ltd, Pretoria, South Africa); and the plants were additionally supplemented once a week with 50 ml of nitrosol fertiliser (Efekto Care (Pty) Ltd).

Plant health was assessed using the LiCor Li-6400XT photosynthesis system (LI-COR Biosciences Inc.). Any plants with photosynthetic values outside one and a half standard deviations of the mean were discarded (Table 3.1). Plants were visually inspected and if plant size was less than average, or if leaves were discoloured, then they were discarded. After three months, a total of 164 of plants were moved to two adjacent phytotrons at the Forestry and Agricultural Biotechnology Institute (FABI) (25° 45' 20.0" S 28° 14' 07.0" E) which were set at a 12 hour photoperiod. The plants were placed in a completely randomised experimental design and allowed to acclimate at 25 ºC for 3 weeks prior to the start of the experiment.

Table 3.1: Photosynthetic carbon assimilation values for all avocado plants.

Rootstock	Day	Mean A ($\mu mol\ m^{-2}\ s^{-1}$)	SD	Mean+ (1.5xSD)	Mean- (1.5xSD)	Numbers removed
R0.09	1	8.58	2.52	12.37	4.80	1
	2	8.03	3.03	12.56	3.49	2
	3	9.08	3.15	13.81	4.35	1
	4	7.62	3.57	12.98	2.26	2
	5	8.37	3.08	12.99	3.75	1
R0.38	1	8.05	1.96	10.99	5.12	1
	2	7.91	2.35	11.43	4.39	3
	3	7.30	2.49	11.03	3.57	1
	4	7.20	2.03	10.25	4.15	2
	5	6.33	3.43	11.47	1.19	2
R0.06	1	8.85	2.46	12.54	5.17	1
	2	7.95	1.99	10.93	4.97	2
	3	8.50	4.50	15.25	1.74	1
	4	8.69	2.19	11.97	5.41	2
	5	8.10	2.98	12.57	3.63	1

The standard deviation (SD) was multiplied by 1.5 to calculate the acceptable range of A. Plants falling outside this range were removed from the trial.

3.2.2 Zoospore Production

For zoospore production, unfiltered V8® vegetable juice (Campbell Soup Company) was decanted into 50 ml falcon tubes and 0.5 g of $CaCO_3$ was added to each tube which was then centrifuged at 4000 rpm at 4 °C for 4 minutes. The pellet was discarded

and the clarified juice was used to make 5 % V8 agar plates and 2 % V8 broth for *Pc* growth. The *Pc* isolates used were GKB4 and HB5, which were obtained from the culture collection of the Avocado Research Programme at the University of Pretoria. In order to restore their virulence, these isolates were grown in 'Granny Smith' apples in an incubator at 25 ºC until lesions developed, and then infected tissue was aseptically excised and inoculated onto 5 % V8 agar plates to allow mycelia to grow. Single hyphal tip isolations were then used to subculture *Pc* onto fresh 5 % V8 agar, where mycelia were allowed to grow. Agar blocks were cut from the mycelial edge and marginally submerged in Petri dishes with 2 % V8 broth and then incubated for 3 days at 25 ºC under fluorescent light. The broth was discarded and the mycelia were washed twice with distilled water, and then placed in fresh river water from a flowing source, which had been filtered twice with Whatman grade one filter paper. The mycelia were returned to the incubator at 25 ºC under fluorescent light until sporangia formed, which took ±3 days. The river water was then discarded and mycelia were pooled into a single container where they were cold shocked for an hour with distilled water at 4 ºC. The pooled mycelial samples were left at room temperature to reach 25 ºC, whereupon they were monitored for zoospore release, and then zoospores were counted. Using a haemocytometer, the zoospore count was measured at 5 x 10^4 ml^{-1}, which was suitable for inoculation.

3.2.3 Infection and Harvest

Root inoculation was done by immersion using two 50 L basins, each placed in a separate phytotron. The control basin contained distilled water, whereas the treatment basin contained a zoospore suspension with a count of 5 x 10^4 ml^{-1}. Plants were uprooted from their media and their root systems were submerged in their respective

basins for an hour, after which they were replanted. Six plants per rootstock type, per time point, were independently harvested (destructively harvested from the total number of plants and thus statistically without replacement). Harvest time points were at 6, 9, 12 and 24 hours post inoculation with *Pc* or mock inoculation with distilled water. For microscopy, three roots with intact tips were randomly selected from each root system and placed in 50 ml falcon tubes in formalin acetic acid alcohol solution (v/v, 1 acetic acid: 1 formaldehyde: 9 ethanol: 9 distilled H_2O) for fixation; and then placed into a cold room at 4 ºC. The remaining root tissue was flash frozen in liquid nitrogen, ground to powder with a tube mill and stored in an ultralow freezer at -80 ºC for use in microplate assays. Ten treated plants from each phytotron were retained to verify the presence or absence of the pathogen.

3.2.4 Verification of Infection

At three days post inoculation, verification plants were harvested and several whole roots per plant were retained for visual identification. The remaining root tissue was flash frozen in liquid nitrogen, ground to powder with a tube mill and stored in an ultralow freezer -80 ºC for the polymerase chain reaction (PCR) protocol. Whole roots were first surface sterilised using 1.5 % bleach solution, cut open and then inoculated onto selective NARPH (nystatin, ampicillin, rifampicin, pentachloronitrobenzene and hymexazol) media (Hüberli *et al.*, 2000). Any colony growth was then further inoculated onto half strength potato dextrose agar (PDA) and allowed to grow and then observed under a Zeiss Axio light microscope (Carl Zeiss Ltd.). Photographs were taken using a CAT S30 smartphone through the microscope eyepiece (Figure 3.1 A, B and C) and growth habits were then observed. Colony isolates from infected plants grown on half PDA produced the characteristic “gnarled” *Pc* growth pattern which was

not seen with uninfected plants, thus confirming that infected plants were indeed infected, whereas uninfected were not.

Deoxyribonucleic acid (DNA) was extracted from frozen root tissue using a modified pine tree root nucleic acid extraction protocol (Chang *et al.*, 1993, Brunner *et al.*, 2001, M^{c}Nickle *et al.*, 2008). An extraction buffer was prepared with Tris and ethylenediaminetetraacetate buffer (TE-buffer), hexadecyltrimethylammonium bromide (CTAB) and polyvinylpyrrolidone (PVP). The extraction buffer was then added to the frozen root tissue, followed by 2-mercaptoethanol (under a fume hood), 5 % polyvinylpolypyrrolidone (PVPP) and spermidine. The mix was incubated at 65 ºC for half an hour, centrifuged and the supernatant was retained for extraction. Two successive chloroform extractions were performed followed by centrifugation. The supernatant was again retained, mixed with chilled isopropanol and kept at -20 ºC overnight. The mix was subsequently centrifuged and the pellet was retained and air dried. The dried pellet containing sample DNA was suspended in TE buffer and then refrigerated for PCR.

The PCR protocol used *Ycin3F-4R* primers which target *Ypt (C2)* and are known for their specificity to *Pc* (Schena *et al.*, 2008, Kunadiya *et al.*, 2017). A PCR mix was prepared using distilled water, DNA polymerase buffer, 5 mM deoxynucleotide triphosphates, 25 mM $MgCl_2$, 10 µM YPT (C2) primers (forward and reverse) and 1U Faststart Taq DNA polymerase. The mix was added to Eppendorf tubes containing the refrigerated sample DNA (15.5 ng/µl), a positive control containing confirmed *Pc* DNA and a negative control containing water. The thermal program was run on an Applied Biosystems™ 2720 Thermal Cycler PCR machine (Applied Biosystems corp.) with the

annealing temperature at 60 ºC. The contents of all tubes were then run on an agarose gel and visualised using GelRed under UV light (Figure 3.1D). The visualised PCR showed infected samples and the positive control shared common bands (300 base pair fragment), whereas the negative control and uninfected samples did not have those bands. This confirms that infected plants were indeed infected and uninfected plants were not infected.

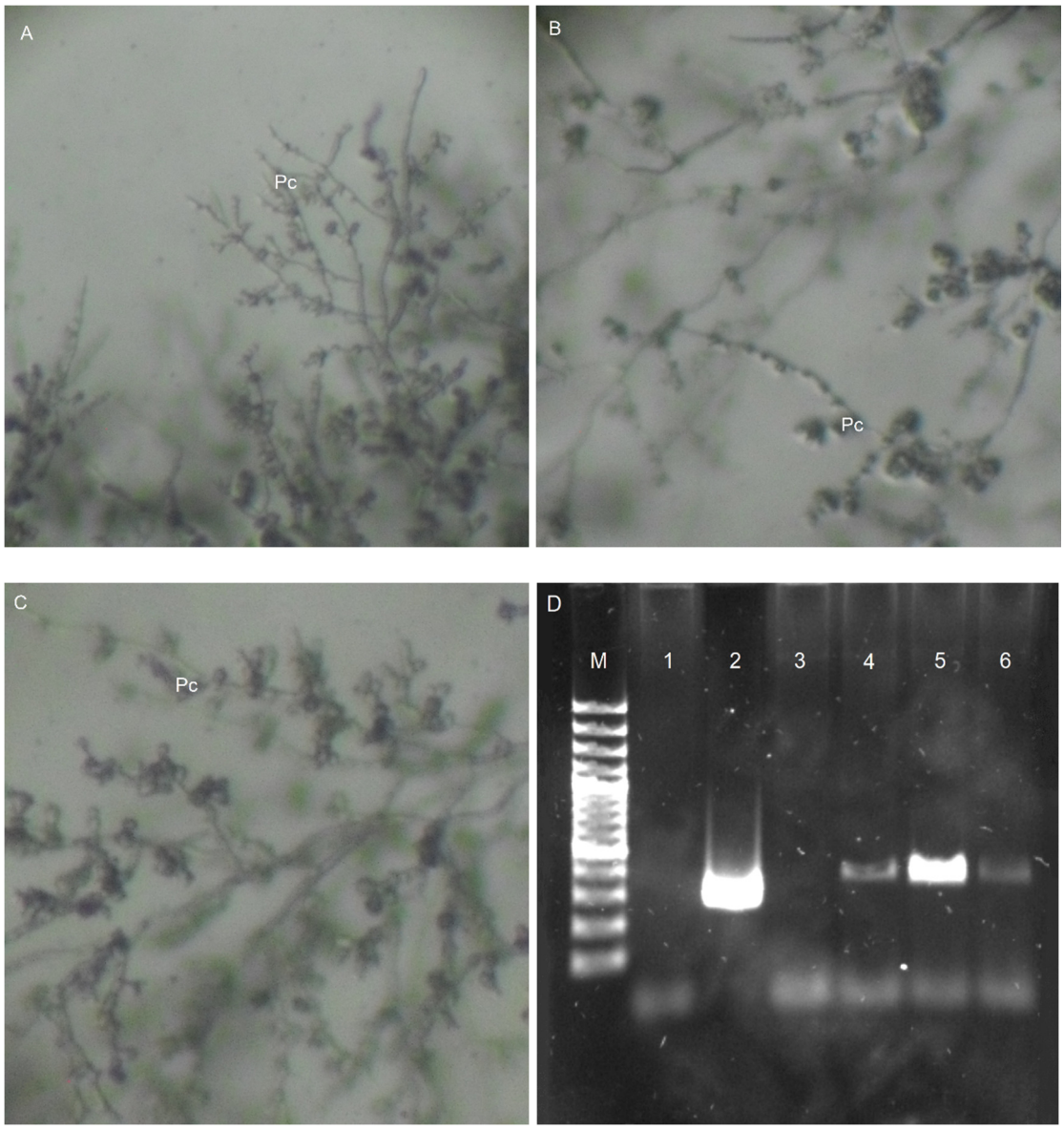

Figure 3.1: Verification of infection. A, B and C: Hyphae (*Pc*) from infected samples growing on half PDA agar with characteristic “gnarled” growth habit. D: Agarose gel visualisation from PCR. M - marker, 1 - negative control (buffer), 2 - positive control (*Pc* sample), 3 - uninfected sample, 4, 5 and 6: infected samples. Infected samples show bands in common with the positive control. The size of the fragment in these bands was 300 base pairs.

3.2.5 Microscopy

The fixed root samples for microscopy were transferred to an oven set at 60 ºC where they were dehydrated using a series of solutions containing butanol, ethanol and water (Table 3.2).

Table 3.2: Dehydration series for microscopy samples.

Series number	**Butanol (%)**	**Ethanol (%)**	**Water (%)**
1	25	30	45
2	40	30	30
3	55	25	20
4	70	20	10
5	85	15	0
6	100	0	0
7	100	0	0

Samples were then embedded with paraffin wax in an oven at 60 ºC until all butanol had evaporated, which took about 4 weeks. Embedded samples were then cast into wax blocks using a Thermolyne Histo-Centre II-N paraffin tissue embedding station (ThermoFisher Scientific Inc.). The blocks were left for 3 days to set, after which they were trimmed, and stored overnight at -20 ºC for wax hardening, and then sectioned at 10 µm using a Reichert-Jung 2040 microtome (Riechert Inc.) All sections in this study were cut transversely and floated on a hot water bath at 40 ºC, then adhered to slides using Haupt's solution and left to dry for 4 days (Bissing, 1974). Dried slides with sections were then dewaxed and rehydrated using a series of solvents in preparation for the calcofluor stain (Table 3.3).

Table 3.3: Slide preparation series.

Series	Contents	Series	Contents
1	100 % xylene	7	100 % ethanol
2	100 % xylene	8	70 % ethanol 30 % water
3	70 % xylene 30 % ethanol	9	50 % ethanol 50 % water
4	50 % xylene 50 % ethanol	10	30 % ethanol 70 % ethanol
5	30 % xylene 70 % ethanol	11	100 % water
6	100 % ethanol	12	0.01 % calcofluor solution

The sections were mounted on slides using a 0.01 % calcofluor fluorescent brightener 28 solution (Sigma-Aldrich Pty. Ltd., product number F3543) and sealed under coverslips with nail varnish. Slides were subsequently left for 12 hours to allow the calcofluor solution to react with the sections. Slides were then viewed and photographed using a Zeiss LSM 510 Meta Confocal Microscope with an Axiocam camera (Carl Zeiss Ltd.) at the Laboratory for Microscopy and Microanalysis, University of Pretoria (25° 45' 10.9" S 28° 13' 45.4" E). The filter sets used for viewing and photography were Zeiss sets 1 (blue emission LP 397 nm for calcofluor staining) and 15 (red emission LP 590 nm for autofluorescence). Finally, image editing was done with Zeiss LSM image browser 4.2. The images recorded from the observations

at all time points were edited, and both red and blue images for each section were compiled into one, in order to give a holistic view of the results.

3.3 Results

3.3.1 Histology

To provide a background for pathological observations, the following was noted. The colour of root cells in all rootstocks was predictable according to the colour of the filter set used to observe them, but their brightness did vary according to the experimental variables of focus and the light emission of particular sections. The epidermis usually appeared red in colour and the hypodermis blue in colour (Figure 3.2 A and B). In the cortex, the colour of cells varied but sclereids appeared blue. In the stele, the endodermis and pith were usually red in colour and the xylem and phloem were blue. The arrangement of xylem varied from tetrarch to octarch.

In the observations of possible defence responses, cell walls that were thickened with lignin were red in colour and were thinner with more variation in thickness, than the blue cell walls that were thickened with callose (Figure 3.2 C and D). The accumulation of phenolics was apparent by red cytoplasm present in some cortical cells (Figure 3.2 C). Artefacts, which were infrequently produced by the staining process, were blue in colour. The hyphae of *Pc* were also blue, but were smaller than artefacts and distinct due to their growth pattern (Figure 3.3). These hyphae were circular when viewed end-on or filamentous-like when viewed side-on. The haustoria-like structures of *Pc* were similar to hyphae and were distinguishable by their protrusion into root cell lumens (Figure 3.3B). Starch grains were observed in some sections (Figure 3.6B), and although they were blue in colour, they could be differentiated from *Pc* hyphae by their

truncated globular shape and specific location against the inner cell wall surface of non-vascular cells.

The wax embedding process caused root samples to become brittle and fragment, making it challenging to cut sections at specific distances from the root tips for observation. Fortunately, it was possible to differentiate sections that were taken further from the tips, as they had more cortical cell wall thickening, more cells with phenolic accumulation, a thicker endodermis and thicker walled vascular cells. This can be seen in Figure 3.2A (closer to the tip) and 3.2C (further from tip). During sectioning, the wax was observed to be pliant which caused cell walls to distort and possibly break, despite precautionary freezing of blocks. This resulted in cell wall fragments (Figure 3.6D) which were blue in colour but were distinguishable from *Pc* hyphae by their angular appearance.

3.3.2 Histopathology

Uninfected control plants showed some cell wall thickening by callose and lignin, the presence of phenolics, and gum deposition in vessels. These observations were used as a standard for comparisons with infected plants, so that only the observations resulting from infection were recorded. At 6 hpi, infected R0.38 (susceptible) sections showed hyphal growth throughout the root, with hyphae almost completely blocking vascular tissue (Figure 3.3). Haustoria-like structures were present in some cells and cell wall thickening with callose was present at the epidermis and cortex (Figure 3.3 and 3.4A and B). The cortex also showed some cells with lignin cell wall thickening and some cells with an accumulation of phenolics. Gum deposition was present in some xylem vessels in the stele. Hyphae were present in some infected R0.06

(resistant) sections and cell wall thickening by callose was evident in the epidermis. Some cortical cells also showed cell wall thickening with lignin in addition to callose (Figure 3.4 C and D). When comparing R0.06 and R0.38, R0.06 exhibited less *Pc* growth. Some R0.06 sections showed more cell wall thickening by callose and lignin than R0.38, but others showed less. Infected R0.09 (resistant) sections did not show any significant differences compared to sections from R0.09 control plants. All of the observations of infected sections from roots harvested at 6 hpi are summarised in Table 3.4.

Table 3.4: Summary of observations from infected root sections harvested at 6 hours post infection with *Phytophthora cinnamomi*.

	Epidermis	**Cortex**	**Stele**	**Xylem**
Infected R0.09	-	-	-	-
Infected R0.06	*Pc*, Bl	Bl, Re	-	-
Infected R0.38	*Pc*, Bl	*Pc*, Bl, Cy, Re	*Pc*	*Pc*, Gu,

Pc: hyphae, Bl: callose cell wall thickening, Re: lignin deposited in cell wall, Cy: phenolic accumulation in the cytoplasm or cell lumen, Gu: presence of gum in the xylem.

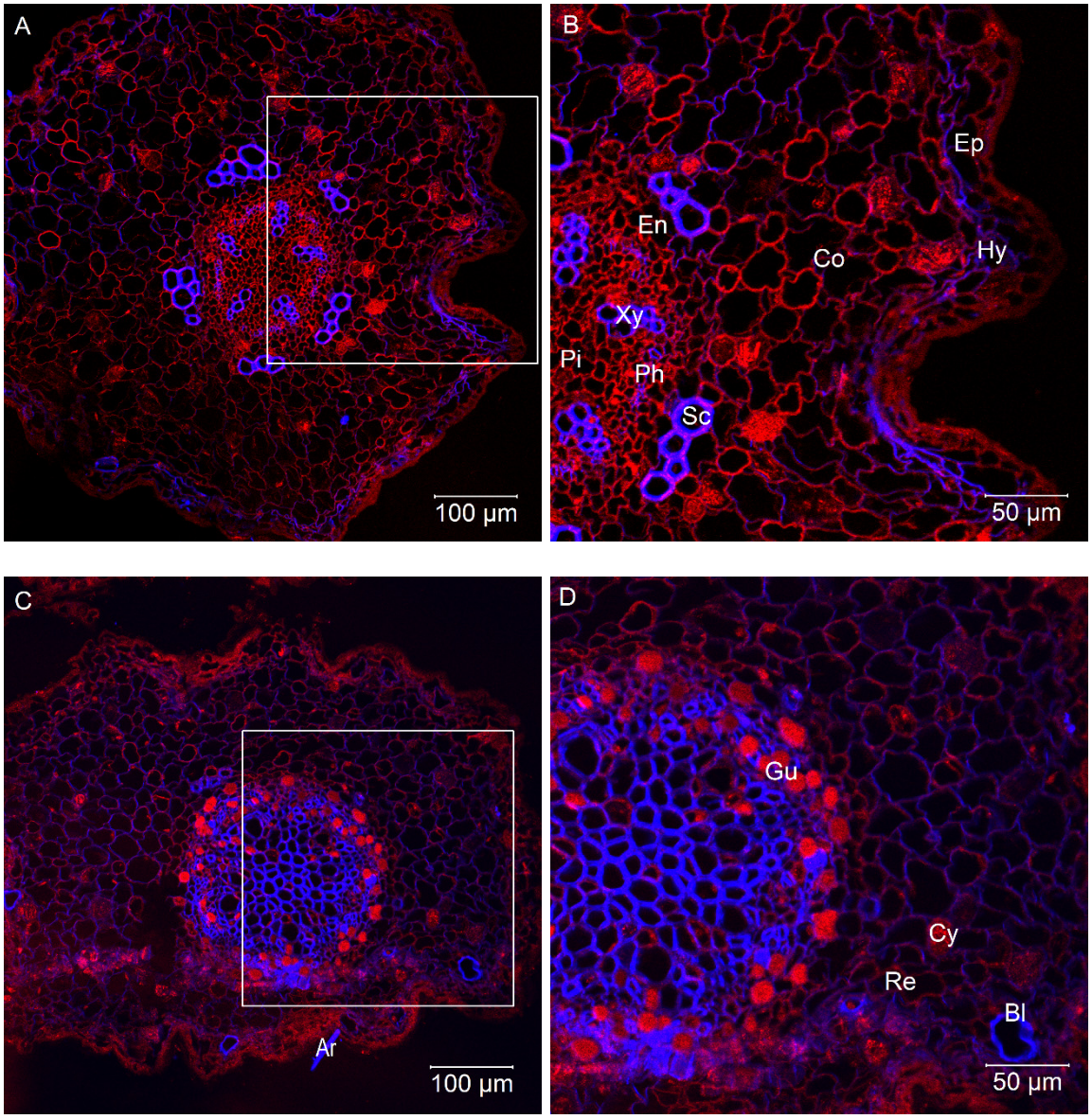

Figure 3.2: Uninfected root sections at 6 hours post infection showing general anatomical observations. A: Whole R0.09 section. B: Magnified area of box in A, with tissue details. Tissue types: epidermis (Ep), hypodermis (Hy), cortex (Co) with sclereids (Sc), stele with endodermis (En), pith (Pi), pentarch xylem (Xy) and phloem (Ph). C: Whole R0.06 section with artefact (Ar). D: Magnified area of box in C with details. These include gum (Gu), cell wall thickening with lignin (Re), cell wall thickening with callose (Bl) and presence of phenolics in the cytoplasm (Cy).

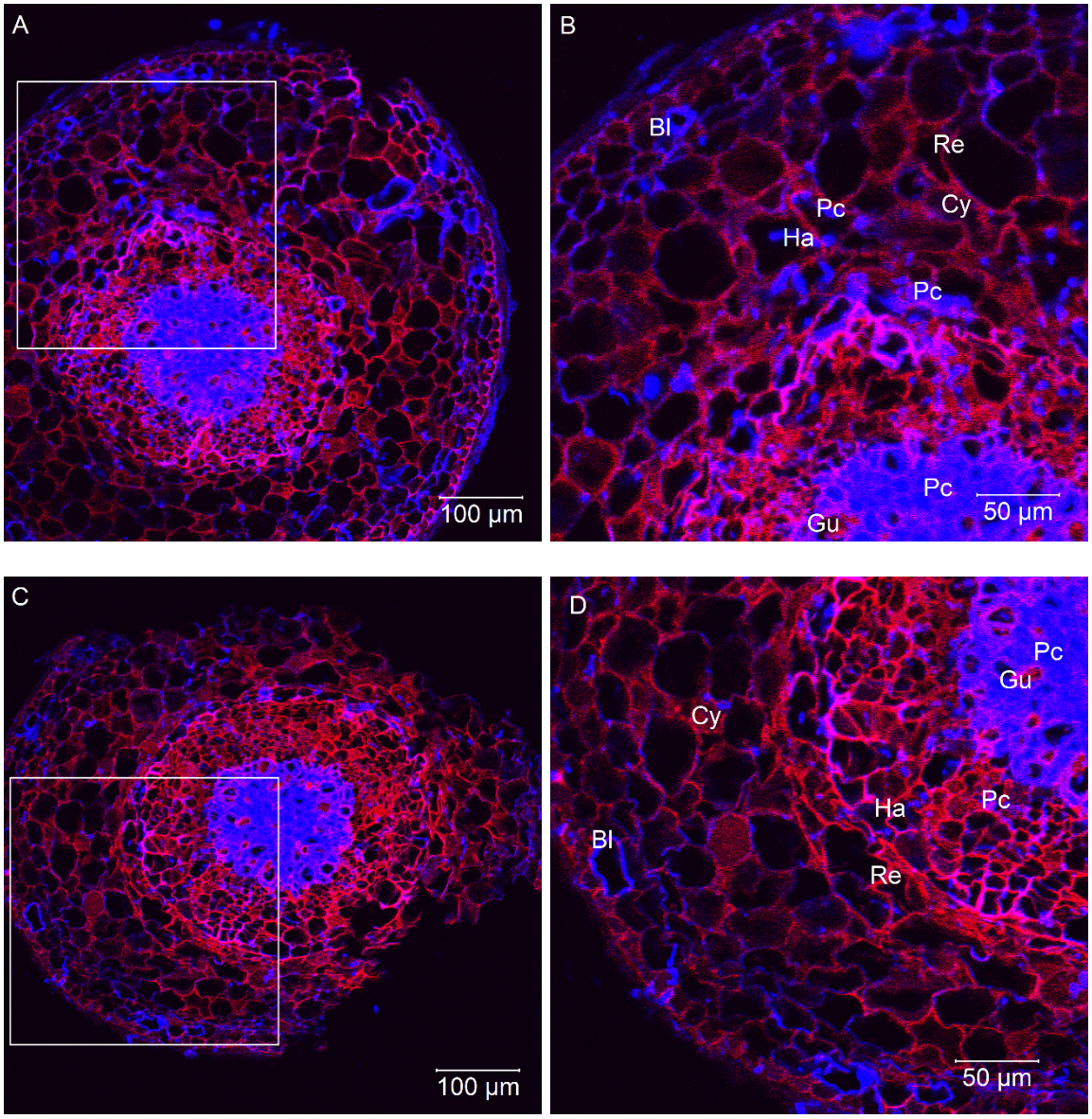

Figure 3.3: Infected R0.38 root sections at 6 hours post infection. A: Whole section showing extent of infection. B: Magnified area of box in A, with details. These include hyphae (*Pc*) (abundant in stele), haustoria like structures (Ha), cell wall thickening with lignin (Re), cell wall thickening with callose (Bl), presence of phenolics in the cytoplasm (Cy) and gum deposits in vessels (Gu). C: Another whole section showing extent of infection. D: Magnified area of box in C, with abbreviations the same as B.

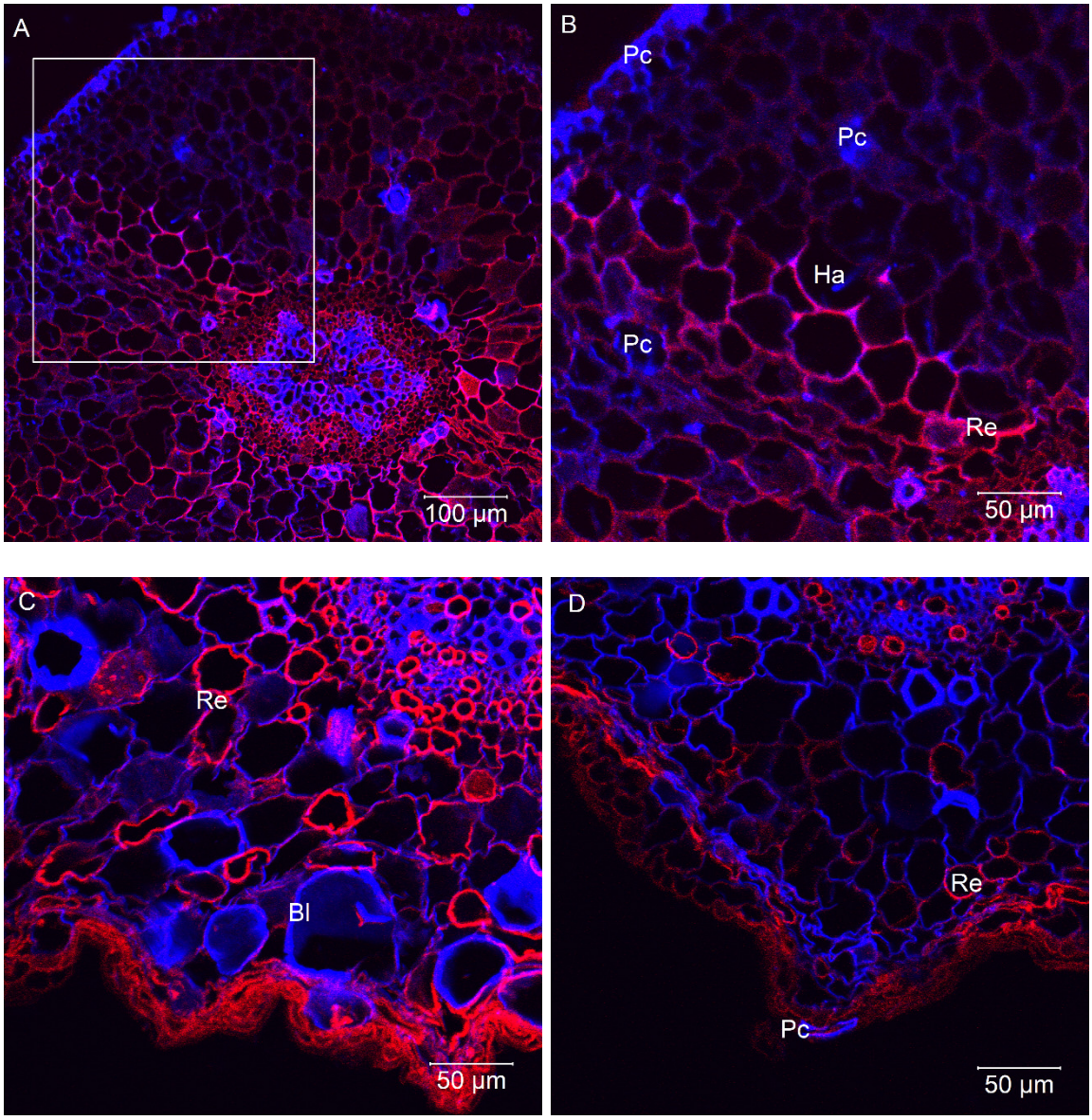

Figure 3.4: Infected root sections at 6 hours post infection. A: A complete R0.38 section showing extent of infection. B: Magnified area of box in A, with details. These include hyphae (*Pc*), haustoria like structures (Ha), cell wall thickening with lignin (Re). C: Infected R0.06 section showing cell wall thickening with callose (Bl) and lignin. D: Infected R0.06 section showing hyphae and cell wall thickening with lignin.

At 9 hpi, infected R0.06 sections showed the presence of *Pc* hyphae in the epidermis of the root, with proximal cell wall thickening by callose and lignin. In the cortex, some cells showed the accumulation of phenolics (Figure 3.5 and 3.6A). Infected R0.38 sections showed some gum deposition in vessels in the stele (Figure 3.6C). Infected R0.09 sections did not show any significant differences compared to the sections from R0.09 control plants. All of the observations of sections from roots harvested at 9 hpi are summarised in Table 3.5.

Table 3.5: Summary of observations from infected root sections harvested at 9 hours post infection with *Phytophthora cinnamomi*.

	Epidermis	**Cortex**	**Stele**	**Xylem**
Infected R0.09	-	-	-	-
Infected R0.06	*Pc*, Bl, Re	Cy	-	-
Infected R0.38	-	-	-	Gu

Pc: hyphae, Bl: callose cell wall thickening, Re: lignin deposited in cell wall, Cy: phenolic accumulation in the cytoplasm or cell lumen, Gu: presence of gum in the xylem.

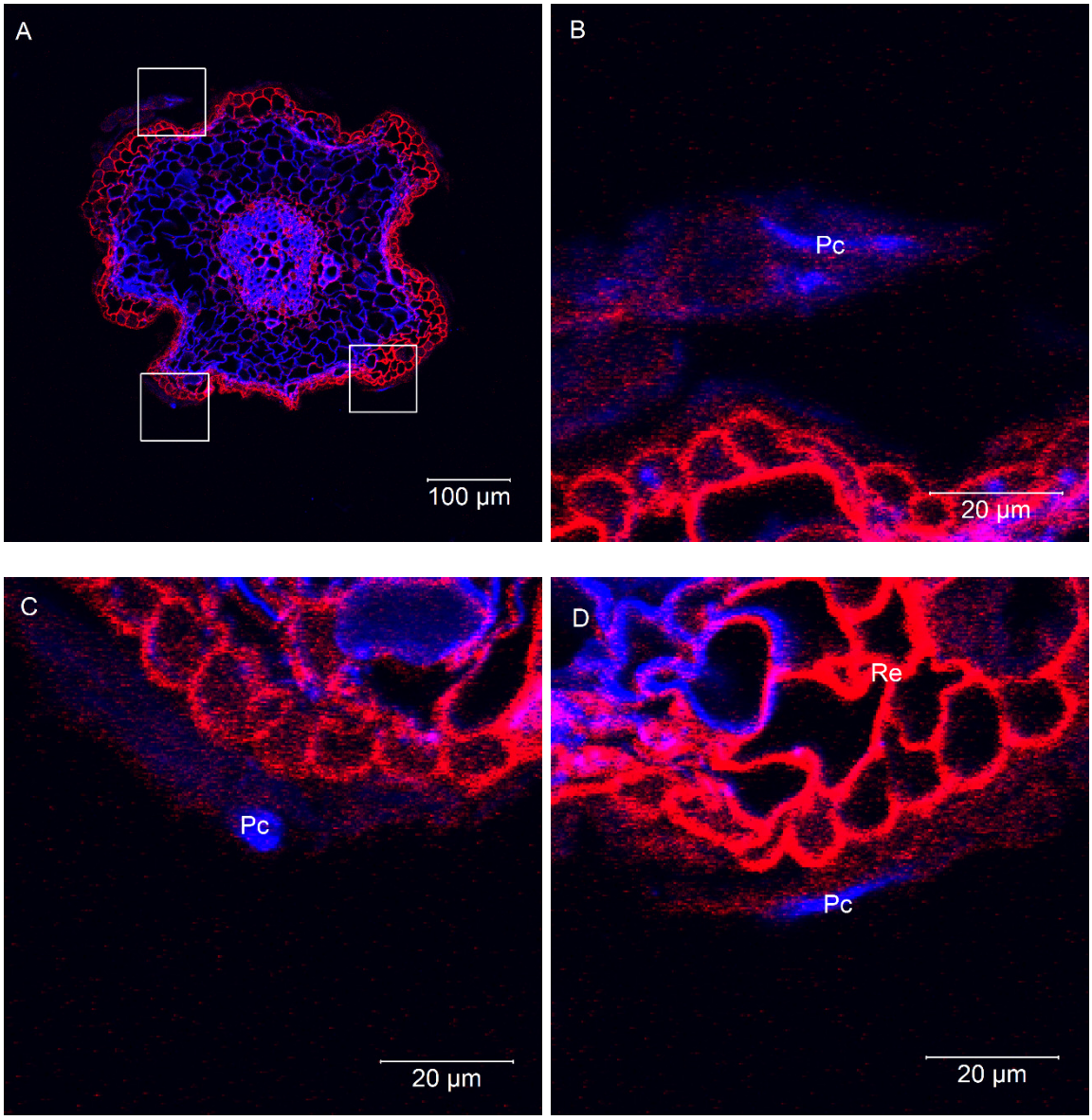

Figure 3.5: An infected R0.06 section at 9 hours post infection. A: A whole section showing sites of infection within white boxes. B: Magnified area of box in A, with hyphae at epidermis (*Pc*). C: Magnified area of box in A, with hyphae. D: Magnified area of box in A, with hyphae and cell wall thickening with phenolics or lignin (Re).

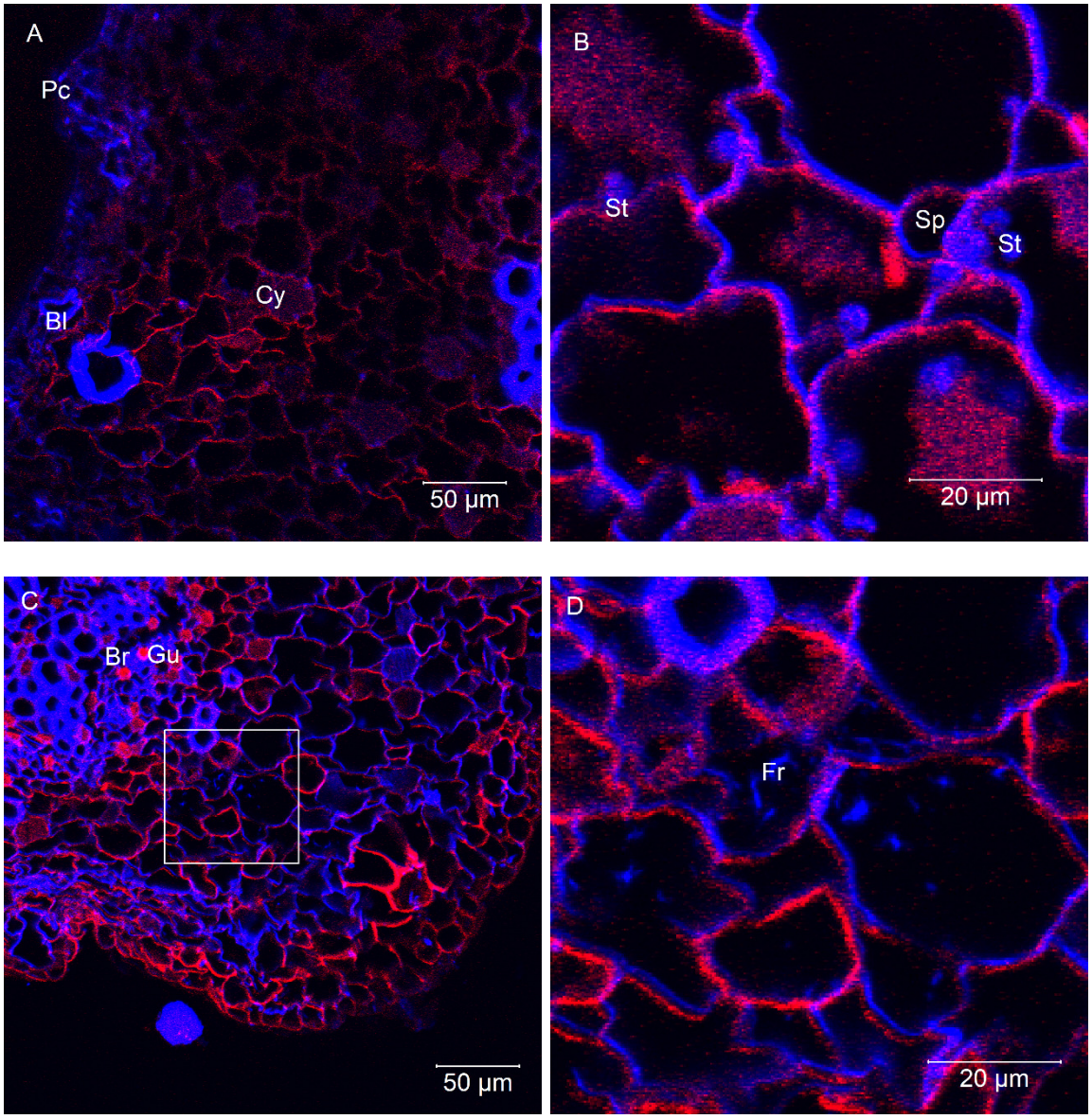

Figure 3.6: Infected sections at 9 hours post infection. A: R0.06 section with epidermal hyphae (*Pc*), epidermal cell wall thickening with callose (Bl) and accumulation of phenolics in cortical cells (Cy). B: Infected R0.09 section showing starch grains (St) and intercellular spaces (Sp). C: Infected R0.38 section with gum deposits in vessels (Gu) and a break in cell walls (Br). D: Magnified area of box in C showing cell wall fragments (Fr)

At 12 hpi, infected R0.09 sections showed the presence of *Pc* hyphae growing throughout the root (Figure 3.7; 3.8A and B). Some cell walls in the epidermis and cortex were thickened with callose and some vessels in the stele showed gum deposits and tyloses. Infected R0.38 sections also showed the presence of tyloses in some vessels of the stele (Figure 3.8D). When comparing R0.38 and R0.09, it was noted that the relative number of tyloses was similar. Infected R0.06 sections showed a high amount of phenolic accumulation in cortical cells compared to other rootstocks (Figure 3.8C). All of the observations of sections from roots harvested at 12 hpi are summarised in Table 3.6.

Table 3.6: Summary of observations from infected root sections harvested at 12 hours post infection with *Phytophthora cinnamomi*.

	Epidermis	**Cortex**	**Stele**	**Xylem**
Infected R0.09	*Pc*, Bl	*Pc*, Bl	*Pc*	*Pc*, Ty, Gu
Infected R0.06	-	Cy	-	-
Infected R0.38	-	-	-	Ty

Pc: hyphae, Bl: callose cell wall thickening, Cy: phenolic accumulation in the cytoplasm or cell lumen, Gu: presence of gum in the xylem, Ty: Tyloses in the xylem.

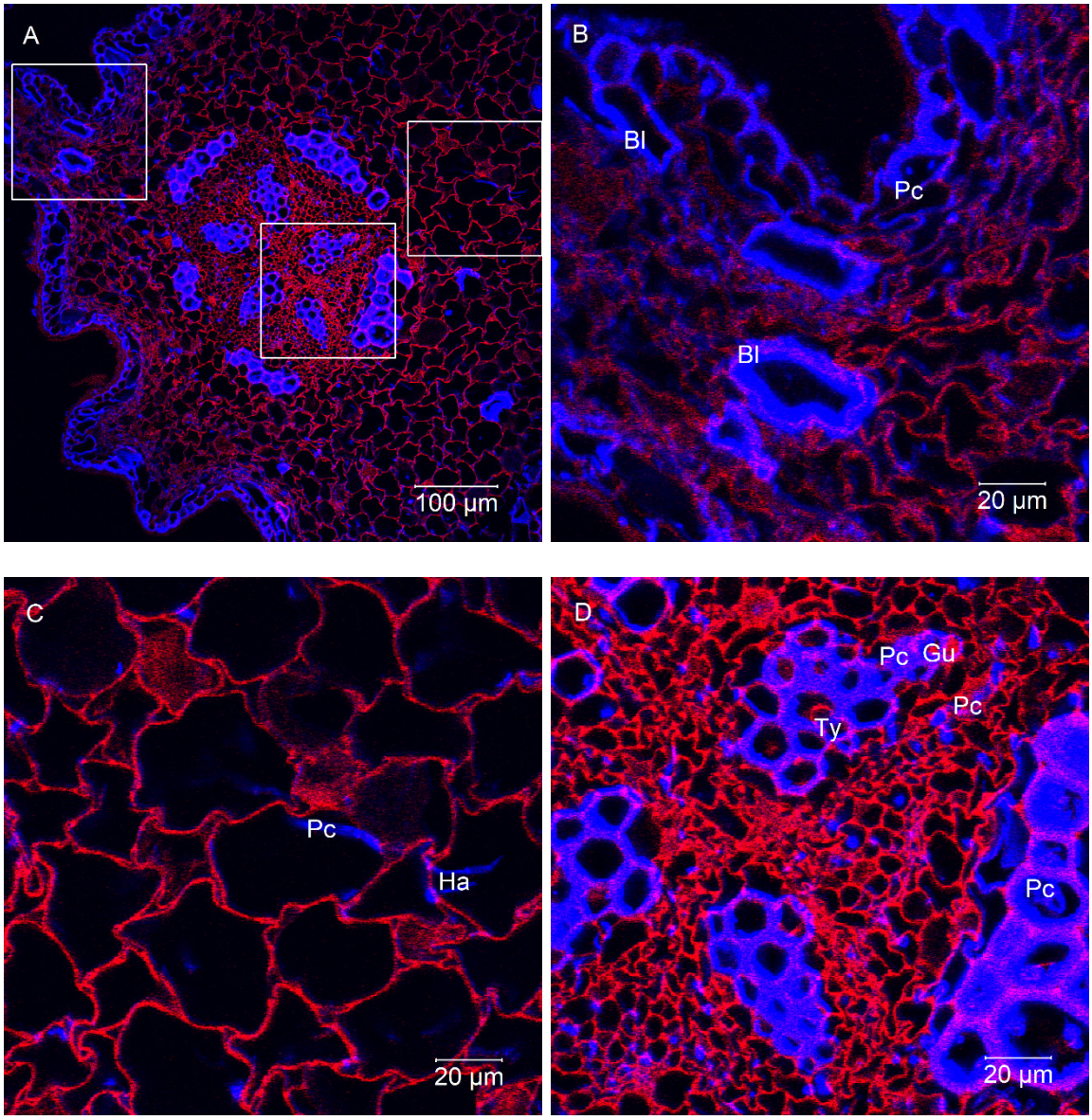

Figure 3.7: A whole infected R0.09 section at 12 hours post infection showing the extent of infection. B: Magnified area of box in A showing epidermal hyphae (*Pc*) and epidermal and cortical cell thickening with callose (Bl). C: Magnified area of box in A showing cortical hyphae and haustoria-like structures (Ha). D: Magnified area of box in A showing hyphae in and around the stele and presence of tyloses (Ty) and gum (Gu) in vessels.

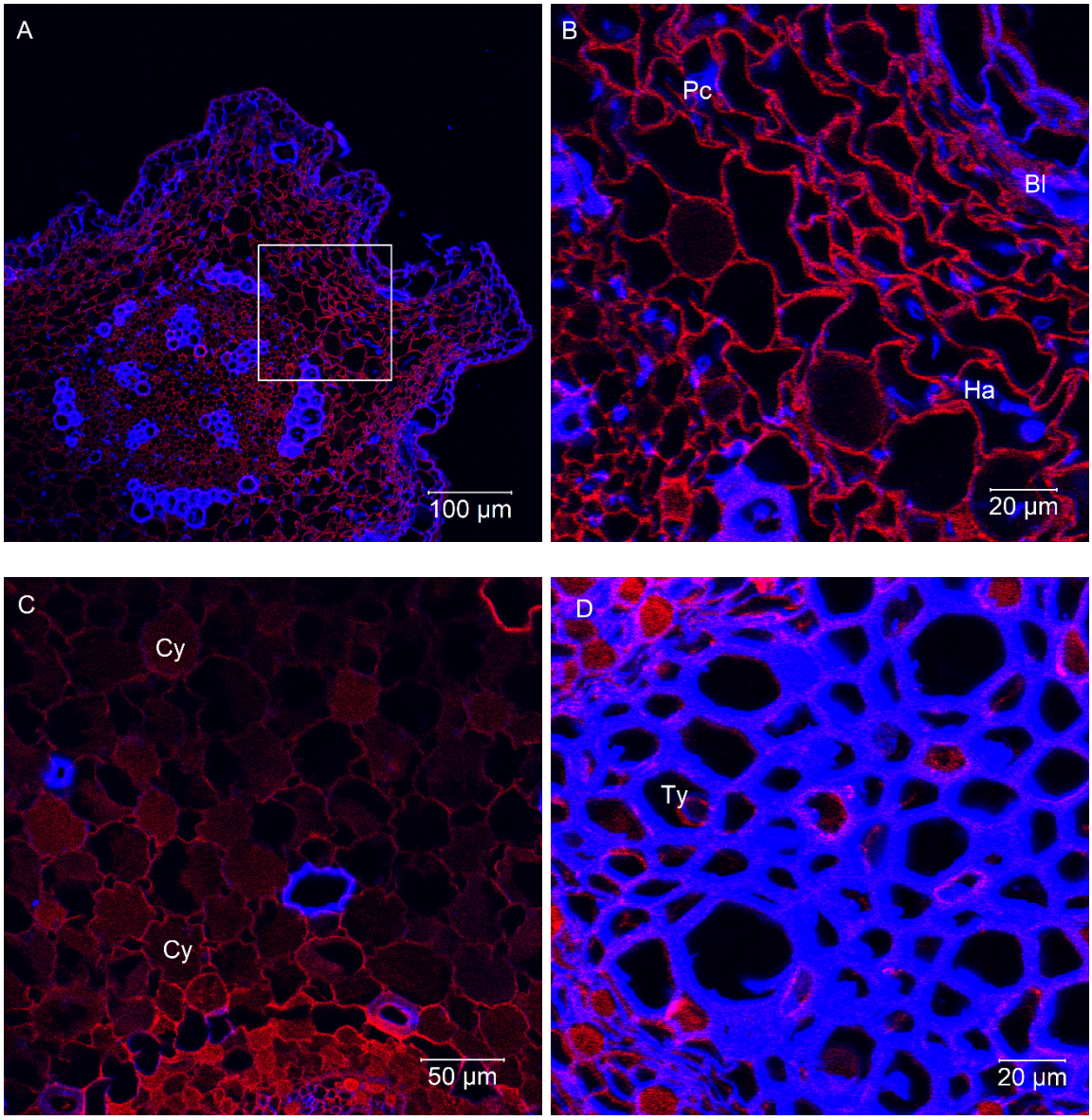

Figure 3.8: Infected sections at 12 hours post infection. A: A whole R0.09 section showing the extent of infection. B: Magnified area of box in A showing cortical hyphae (*Pc*), haustoria like structures (Ha) and epidermal cell wall thickening with callose (Bl). C: R0.06 section showing accumulation of phenolics in cortical cells (Cy). D: R0.38 section showing a tylosis in a vessel (Ty).

At 24 hpi, tyloses in vessels were observed in infected R0.09 and R0.38 sections (Figure 3.9 A and B), which were relatively similar in number. No significant observations due to infection were seen in R0.06. All of the observations of sections from roots harvested at 24 hpi are summarised in Table 3.7.

Table 3.7: Summary of observations from infected root sections harvested at 24 hours post infection with *Phytophthora cinnamomi*.

	Epidermis	**Cortex**	**Stele**	**Xylem**
Infected R0.09	-	-	-	Ty
Infected R0.06	-	-	-	-
Infected R0.38	-	-	-	Ty

Ty: tyloses in xylem.

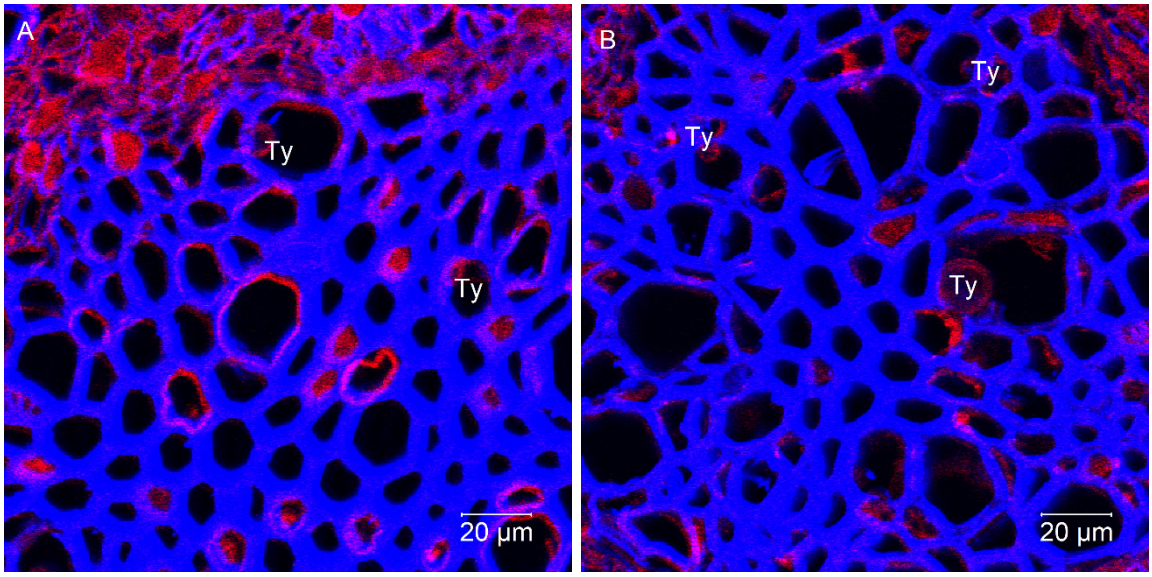

Figure 3.9: Infected sections at 24 hours post infection. A: R0.09 section of stele showing tyloses in vessels (Ty). B: R0.38 section of stele showing tyloses in vessels.

3.4 Discussion

This study used confocal microscopy to identify histopathological differences in the susceptible R0.38 and resistant R0.06 and R0.09 avocado rootstocks in the first 24 hours after infection with *Pc*. These differences were specifically observed in respect to cell wall thickening by callose and lignin, accumulation of phenolics in cells and the presence of gum and tyloses in vessels.

At the earliest time point of 6 hpi, it was noticeable that hyphal growth in the susceptible R0.38 was observed as far into the root as the stele, whereas the resistant R0.06 only showed hyphal growth into the epidermis and the resistant R0.09 showed no hyphal growth. This early penetration could demonstrate the known susceptibility of R0.38 and the resistance of R0.06 and R0.09. However, R0.09 later showed hyphae growing as far as the stele at 12 hpi, which was unusual, as complete penetration was unexpected for a resistant rootstock. It is also surprising in this study that hyphal penetration occurred faster in general, in comparison to a confocal study done by van den Berg *et al.* (2018). In that study, hyphae were first detected in R0.06 at 6 hpi, but they did not grow further than the epidermis by 24 hpi. Although R0.38 and R0.12 differ as roostocks, hyphae in suceptible R0.12 only reached the stele by 24 hpi, in contrast to R0.38 which showed hyphae in the stele at 6 hpi. A possible explanation for the faster penetration could be that after infection, the previous study replanted rootstocks only in vermiculite, but in the present study, an abrasive vermiculite and perlite mix was used (which was noticably abrasive on one’s skin), which could have caused small root epidermal lesions, thus allowing *Pc* easier access to root tissue and resulting in faster colonisation.

Callose thickening of cell walls was hypothesised to occur earlier, or in greater quantities in resistant rootstocks, but this could not be confirmed because, in terms of timing, both the susceptible R0.38 and resistant R0.06 produced early epidermal and cortical callose thickening at 6 hpi. The amount of callose associated with resistance was inconclusive as some R0.06 sections showed an increase in callose deposition compared to R0.38, but others showed a decrease. The R0.09 rootstock only showed increased callose deposition at 12 hpi, which is later than 6 hpi, and thus resistance was not associated with early callose deposition. While bearing in mind that R0.09 and R0.06 differ, the results are in contrast with van den Berg *et al.* (2018), which associated callose deposition with resistance to *Pc*, as the resistant R0.06 produced callose by 6 hpi, but the susceptible R0.12 did not produce callose in the first 24 hpi. Differences in early callose deposition in response to infection are analysed in the next chapter using a quanititative perspective to further test this hypothesis.

Lignin thickening of cell walls was hypothesised to occur earlier, or in greater quantities in susceptible rootstocks, but this could not be confirmed as both the resistant R0.06 and susceptible R0.38 rootstock produced early cortical lignin thickening in response to infection at 6 hpi. The amount of lignin associated with resistance was inconclusive as some R0.38 sections showed an increase in lignin deposition compared to R0.06, but others showed a decrease. The R0.06 rootstock also produced lignin thickening at 9 hpi as a result of infection, which did not associate lignin with susceptibility. These results contrast with van den Berg *et al.* (2018), where R0.06 produced no lignin thickening in response to infection, and lignin was only seen in the susceptible R0.12 (bearing in mind that R0.12 and R0.38 differ), suggesting that lignin was associated with susceptibility. Differences in early lignin deposition in response to infection are

analysed in the next chapter using a quanititative perspective to address the contrast and further test this hypothesis.

Phenolic accumulation in cells was hypothesised to occur earlier, or in greater abundance in resistant rootstocks but this was disproved as a relatively large amount of phenolics were first seen, as early as 6 hpi, in the cortical cells of susceptible R0.38, and only later seen in the resistant R0.06 at 9 hpi and 12 hpi. In comparison, a similar study observed the autofluorescence of phenolics and tannins at time points of 24 and 48 hpi in the roots of a moderately field resistant 'Duke 7' rootstock, (Phillips *et al.*, 1987). Although the present study used a different resistant rootstock and at earlier time points compared to Phillips *et al.* 1987, the lack of phenolics from R0.09 and R0.06 at 24 hpi could be attributed to the fact that it was difficult to obtain sections from precise distances from the root tip. Thus observations from different parts of the root may have been made. As high numbers of zoospores are known to adhere to the root surface between 1 and 4 mm behind the root tip (Zentmyer 1980), viewing sections from outside this zone could result in missing important observations.

For tyloses and gums, their earlier presence and greater abundance in vessels was hypothesised to be associated with susceptible rootstocks. This was proven for gum deposits which were first seen in the susceptible R0.38 as early as 6 hpi, whereas resistant rootstocks only later showed gum deposits at 12 hpi. As similar numbers of tyloses were first observed in the vessels of both resistant R0.09 and susceptible R0.38 at 12 hpi, it could not be proven that the growth of tyloses or their abundance were associated with susceptibility. This is in contrast to the study by van den Berg *et al.* (2018), who found that the resistant R0.06 showed the presence of tyloses at 12

hpi, but the susceptible R0.12 showed tyloses as early as 3 hpi (noting that R0.38 and R0.12 differ), suggesting that tyloses were associated with susceptibility. The differences between these studies could be attributed to the fact that it was difficult to obtain sections from precise distances from the root tip.

The first confocal examination of R0.38 showed the production of proximal cell wall thickening by callose and lignin deposition, high levels of phenolics inside of cells and gums and tyloses in vessels in response to infection. Despite these responses, it was confirmed susceptible to *Pc*, as hyphae were able to penetrate the root as far as the stele by 6 hpi, which was earlier than resistant rootstocks.

There were experimental limitations to the present study. Firstly, as the wax embedding process was aggressive on the root tissue, the roots became fragmented which made it difficult to observe sections at precise distances from the root tip, which may explain the lack of hyphal observations at 24 hpi (Zentmyer 1980). However, there was still some comparability between samples as sections further from the root tip had a larger diameter and were more differentiated. The wax embedded samples were also brittle which caused distortion and cell wall breaking in some samples. The use of JB-4 resin instead of wax for future studies could possibly avoid root fragmentation and section fragility. Secondly, control plants showed a degree of cell wall thickening by callose and lignin, which is thought to be due to mechanical stress caused by handling during the trial, as suggested by Chen and Kim (2009) and Trupiano *et al.* (2012). To exclude the possibility of mechanical stress, the use of a media-free growing system is recommended (Neilsen, 2016). However, such a system needs to be carefully optimised to avoid flooding stress, to which avocado is rather prone

(Ploetz and Schaffer, 1989). Lastly, microscopy provides spatial information and high resolution in observations, but this is limited to only a very thin section of the total tissue. This fact makes it easy to miss important observations; therefore, the use of quantitative studies to support microscopy work could be helpful. For more accurate microscopy studies, the use of transformation, gene knock-outs and the use of fluorescent markers could allow responses to infection to be more easily examined (Gilroy *et al.*, 2014).

For further research, earlier time points could be examined to determine when hyphae penetrate the root epidermis and to show the resulting defence responses in the roots. The production of tyloses and gums and their effect on the spread of *Pc* through vascular tissue could further be examined using longitudinal sections in microscopy. Lastly, R0.09 could be investigated to understand if hyphae normally grow pervasively in its roots, which would be unexpected for a resistant rootstock, and if they do, then R0.09 could be examined to understand the mechanism of its resistance.

3.5 Conclusion

The use of confocal microscopy to observe differences in the defence response of resistant and susceptible avocado rootstocks infected with *Pc* provided some insights. The early pervasive hyphal growth into the stele seen in R0.38 was consistent with its susceptibility and the hyphal growth limited to the epidermis in R0.06 was consistent with its resistance. However, the pervasive hyphal growth at 12 hpi seen in R0.09 was not expected for a resistant rootstock, and future investigations using earlier time points could help to understand the mechanism of its resistance. It was also noted that the rate of hyphal progression into the tissue of rootstocks appeared to be accelerated

and this may have been facilitated by the abrasive media which could have caused lesions and therefore points of entry for the pathogen.

By using microscopy it was not possible to determine whether the earliness or amount of callose deposition was associated with resistance, nor could the earliness or amount of lignin be associated with susceptibility. However, the next research chapter analyses callose and lignin quantitatively to retest these hypotheses. The early and sizable increase in phenolics seen in susceptible R0.38 suggests that phenolic accumulation was not associated with resistance. As expected, the early deposition of gum was possibly associated with susceptibility, but not with resistance. Unfortunately, the growth of tyloses could not be linked to susceptibility as they were present in visually equal quantities in both the susceptible and resistant rootstocks. Future studies may benefit from the use of longitudinal rather than transverse sections to better observe the vascular progression of *Pc* and the growth of tyloses.

3.6 References

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Chapter 4

Quantitative Biochemical Differences in the Response of Avocado Rootstocks to Infection with *Phytophthora cinnamomi*

4.1 Introduction

Quantitative assays and microscopy studies have been used to examine the defence responses of avocado to infection with *Phytophthora cinnamomi* (*Pc*) (Phillips *et al.*, 1987, van den Berg *et al.*, 2018). Microscopy, which was used in the previous chapter, can observe the specific location of defence responses, but it is only capable of examining limited amounts of root tissue and therefore it can miss important histochemical changes in response to infection. However, microscopy can be complemented by the use of quantitative assays which examine a larger tissue volume. This chapter focuses on quantitative assays which use fluorescence to analyse callose production, and absorbance to analyse the production of lignin, reactive oxygen species (ROS) and total antioxidants. Plant detection of pathogens initiates the production of ROS and a signal cascade inducing the transcription of inducible defence related genes, including those involved with callose and lignin deposition to strengthen cell walls against entry (Jones and Dangl, 2006, Daudi et al., 2012, Eckardt, 2017). The production of apoplastic ROS plays a vital role in transmitting signals to prepare other plant cells for defence but also oxidises monolignols into the polymer lignin in cell walls (Barros *et al.*, 2015, Torres, 2010). The production of ROS is then tempered by antioxidants to prevent oxidative damage and to stop ROS signal propagation (Gill and Tuteja, 2010, Torres, 2010). For this chapter, it was hypothesised that the earlier or increased production of callose, ROS and antioxidants would be associated with resistance and that an earlier or increased production of lignin would be associated with susceptibility. To test these hypotheses, the aim of the chapter was to compare the early infection response of resistant and susceptible rootstocks, and the objective was to quantitatively observe the defence responses in the first 24 hours post inoculation (hpi) in the various rootstocks. The

quantification of callose, lignin, ROS and total antioxidants in response to infection was performed for the first time on R0.38, R0.09 and R0.06 rootstocks.

4.2 Methods and Materials

4.2.1 Plant Material

As described in Chapter 3, six plants per clonal rootstock type (R0.06: resistant, R0.09: resistant and R0.38: susceptible), per time point, were independently harvested without replacement at 6, 9, 12 and 24 hpi after inoculation with *Pc* or mock inoculation with distilled water. Root tissue was flash frozen in liquid nitrogen, ground to powder with a tube mill, and then stored in a -80 ºC ultralow freezer for assay use.

4.2.2 Microplate Assays

Fluorescence and absorbance assays were performed using a SpectraMax Paradigm multi-mode microplate reader (Molecular Devices (LLC)), which used black or translucent corrosion resistant polypropylene microplates respectively. The excitation, emission and absorbance wavelengths were optimised as required for each assay. In order to avoid the presence of particulate matter during readings, pipetting was performed in a laminar flow except when volatiles were used, which required a fume hood. Additionally, all reagents were prepared with distilled and filtered water from a Merck Millipore Simplicity® purification system (Merck KGaA) and furthermore, non-corrosive reagents were filtered with Whatman grade one filter paper. Six biological replicates were used for each rootstock type per time point and four technical replicates were used per sample, blank or standard. Each microplate reading included both blanks and standards, for the correction of background interference and sample quantification, respectively. The preparation of these was assay specific and is

explained in due course. For accuracy, multiple reads were taken per well, and the data was processed and recorded for analysis using SoftMax Pro software, version 6 (Molecular Devices (LLC).

4.2.3 Data Analysis

Using IBM® SPSS® Statistics v23 (SPSS Inc.), the data was analysed to ensure that it met the assumptions for analysis of variance (ANOVA) and independent *t*-tests (Chapter 6: Appendix). The data was then analysed by ANOVA using the full factorial model at a significance level of $\alpha = 0.05$. As overall significance of the corrected model was significant for all treatments, independent *t*-tests were performed as a specific pairwise comparison between infected and uninfected means at each time point, at a significance level of $\alpha = 0.05$. Thus, the uninfected rootstocks were used solely as controls to eliminate the amount of compound of interest produced from metabolic and possibly from handling or environmental sources, in order to strictly consider the amount of compound produced only in response to infection. This amount is referred to as the “difference in means” as it is the difference between the means of infected and uninfected rootstocks of the same type, at each time point. The statistically significant differences in means were then used to compare the response to infection of different rootstock types at the same time point and the same rootstock type across time points. Significant differences in means were then used to observe and analyse the overall pattern of change between rootstock types over the course of infection.

4.2.4 Callose Assay

The assay used aniline blue as a fluorophore, which reacted with the β-glucan callose to produce a complex that is excited by light at the wavelength of 410 nm and

subsequently emits fluorescence at 460 nm (Evans *et al.*, 1984). This fluorescence was measured and compared to the fluorescence of a known quantity of complexed paramylon β-glucan standard in order to determine the amount of callose in the samples (Shedletzky *et al.*, 1997). The assay was performed according to Hirano *et al.* (2006) and Voigt *et al.* (2006), with the necessary modifications for avocado roots. Optimisations were performed on the pH of the reaction mix to maintain complex stability while decolourising excess dye; the dilution of samples to prevent fluorescence saturation; the amount of polyvinylpolypyrrolidone (PVPP) added to remove interfering phenolics while minimising fluorescence interference and the construction of a standard curve (Figure 4.1) using a paramylon standard (Sigma-Aldrich Pty. Ltd., product number 89862).

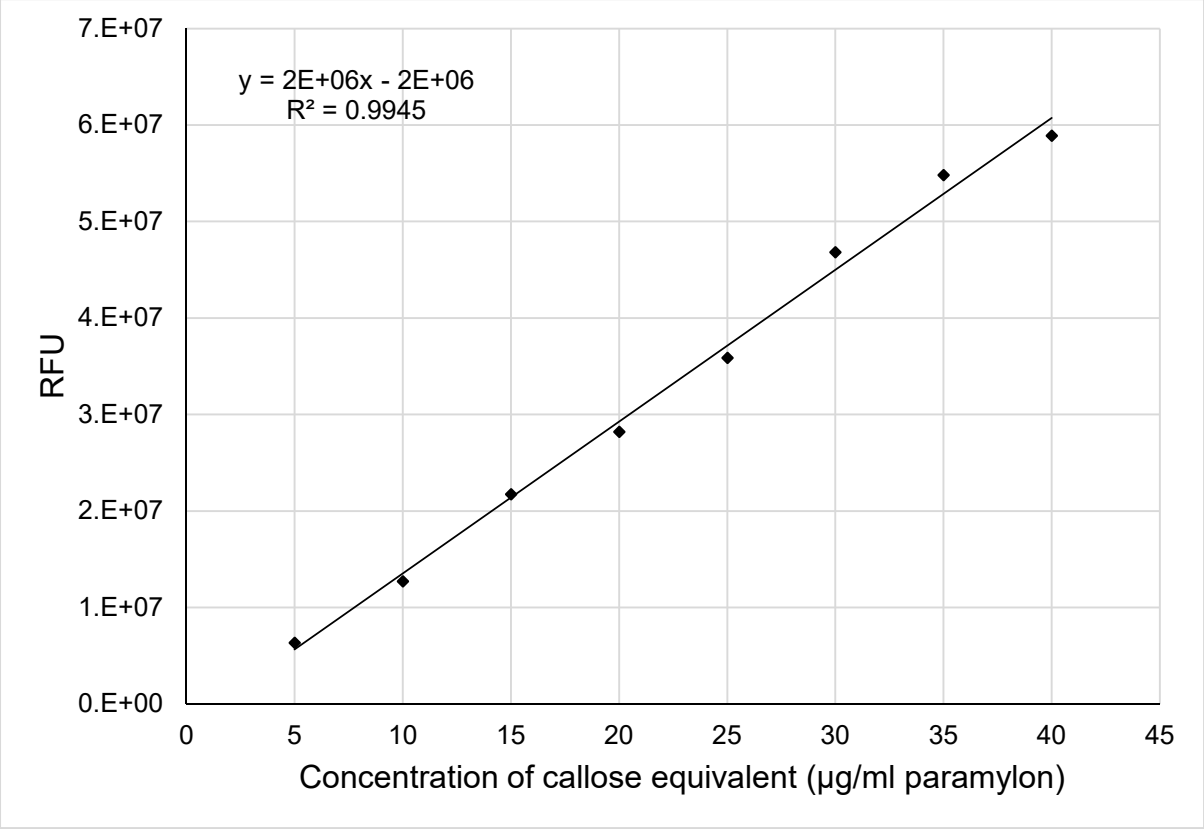

Figure 4.1: Callose standard curve. Fluorescence was measured in relative fluorescence units (RFU). Callose content of samples was estimated using their fluorescence against the curve generated by the β-glucan standard, paramylon.

The frozen powdered root samples were thawed and 0.05 g of each sample was mixed with 0.05 g PVPP. They were then washed and centrifuged at 3000 rcf twice with 96 % ethanol at 50 ºC for 5 minutes to remove compounds which cause fluorescence interference (such as phenolics). The remaining pellet was dried and then suspended in 1 ml of 1 M NaOH at 80 ºC for 15 minutes to hydrolyse the callose. The hydrolysed mix was centrifuged for 15 minutes to pellet out solids, and the supernatant was then retained for analysis. The supernatant was diluted with 1 M NaOH, as optimised and then mixed in tubes, either with aniline blue dye mix (Sigma-Aldrich Pty. Ltd., product number B8563), which was prepared according to Shedletzky *et al.* (1997); or with a

blank dye mix (no aniline blue) to estimate background interference. Standards were prepared in tubes using paramylon and mixed with the aniline blue dye mix. All tubes were heated at 50 ºC for 20 minutes and allowed to cool to room temperature for 20 minutes, while they were mixed to decolourise the excess dye. All mixes were pipetted into a microplate and fluorescence readings were taken at 460 nm, after excitation at 410 nm. The amount of callose in µg/ml of paramylon equivalents in blank adjusted samples was determined by a standard curve that was generated by standard readings.

4.2.5 Lignin Assay

Digested lignin content of a sample can be measured at an absorbance of 280 nm and quantified by comparison with the absorbance of a known quantity of lignin standard. The acetyl bromide assay for lignin was performed according to Moreira-Vilar *et al.* (2014), but with the required alterations for avocado roots. Optimisations were performed on sample dilutions to prevent absorbance saturation, as well as on the construction of a standard curve (Figure 4.2) using alkali lignin (Sigma-Aldrich Pty. Ltd., product number 471003). The ground and frozen avocado root samples were placed in an oven at 55 ºC and were dried until they reached a constant mass. From each sample, 0.06 g was weighed and placed in a polypropylene Eppendorf tube, washed, and centrifuged at 14000 g for 5 minutes with 1.5 ml of 50 mM potassium phosphate buffer at pH 7, 1.2 ml of Triton X-100, 1.2 ml of 1 M NaCl solution, 0.8 ml of acetone and 1.2 ml of distilled water to remove interfering compounds. The samples were then dried again at 55 ºC, until they reached a constant mass, digested with 25 % acetyl bromide in glacial acetic acid at 70 ºC for 1 hour, cooled and allowed to precipitate overnight. The pellet was discarded and 0.115 ml of 2 M NaOH and 0.115

ml of 5 M hydroxylamine HCl were added to the supernatant for stabilisation. The blanks were also prepared using NaOH and hydroxylamine HCl, but without any root material or extract, and the standards were prepared only with alkali lignin. The samples, blanks and standards were pipetted into microplates and absorbance was measured at 280 nm. Lastly, the lignin content in mg/ml in blank adjusted samples was determined by a standard curve, which was generated by the standards.

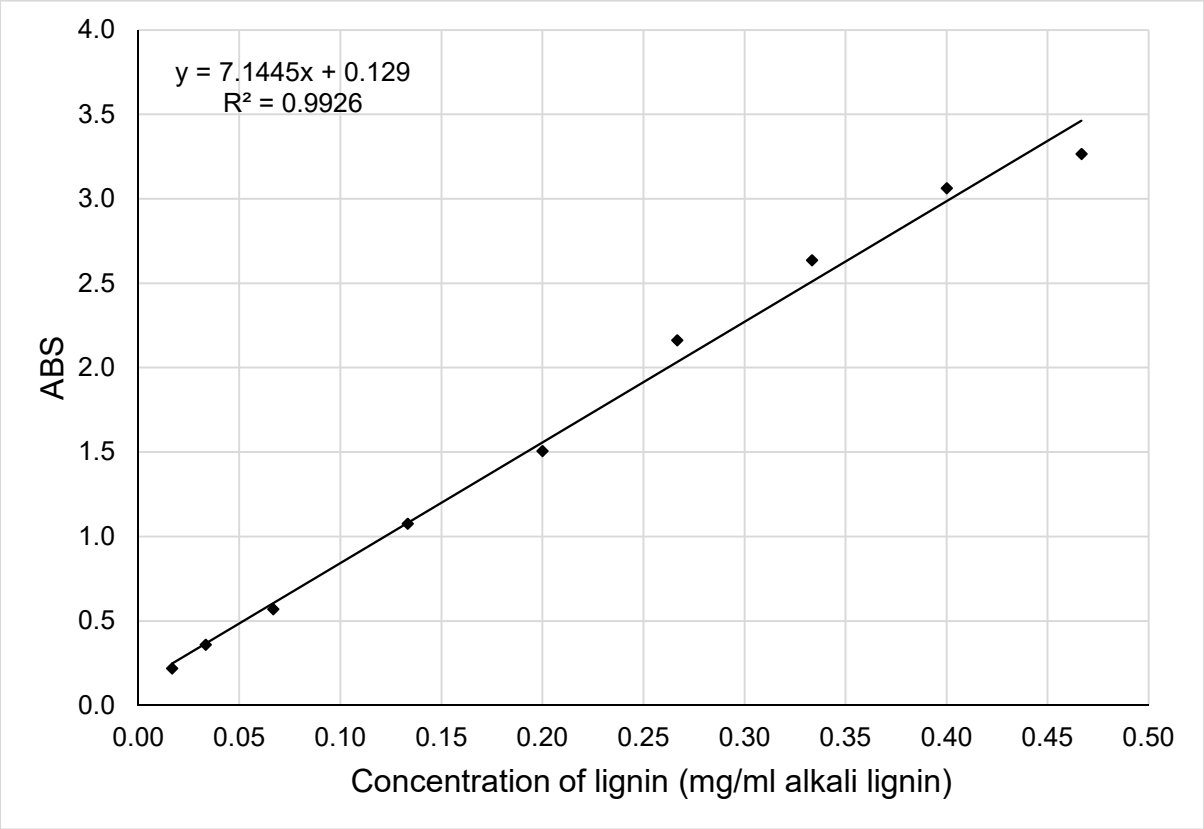

Figure 4.2: Lignin standard curve. Absorbance is abbreviated as ABS. Lignin content of samples was estimated using their ABS against the curve.

4.2.6 Reactive Oxygen Species Assay

The assay was performed according to Hodges *et al.* (1999) and Zheng *et al.* (2006), but with the needed modifications for avocado roots. Cellular lipid peroxidation by ROS

produces malondialdehyde (MDA), which can react with thiobarbituric acid (TBA) in the presence of trichloroacetic acid (TCA) to produce a coloured compound that can be quantified by measuring its absorbance at 532 nm. Optimisations were carried out on the amount of PVPP required to remove the interfering phenolics; on sample and standard dilutions to prevent absorbance saturation; which facilitated the construction of a standard curve (Figure 4.3) using MDA salt (Sigma-Aldrich Pty. Ltd., product number 63287).

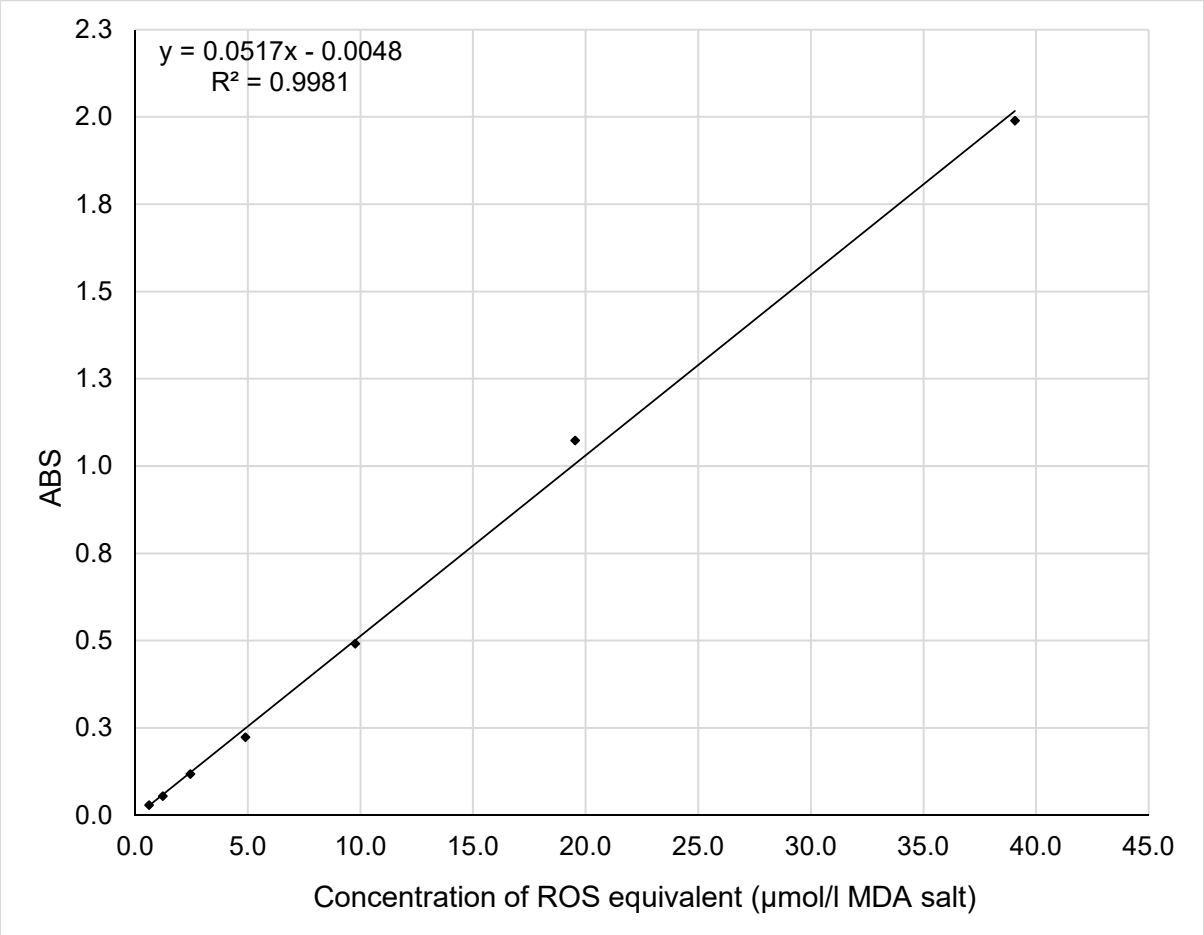

Figure 4.3: Reactive oxygen species standard curve. Absorbance is abbreviated as ABS and ROS was measured in ROS equivalents of MDA salt in µmol/l.

For the assay, two reaction mixtures were prepared: +TBA (0.65 % TBA in 20 % TCA) and –TBA (20 % TCA only). The +TBA was used to prepare samples for absorbance measurement, but the –TBA was used to produce blanks in order to correct for potentially unwanted background absorbance. From each ground and frozen avocado root sample, 0.2 g was weighed and mixed with 0.03 g PVPP in 1.77 ml of 80 % ethanol and then centrifuged at 12000 g for 15 minutes to remove interfering compounds, which were pelleted. The supernatant was retained on ice and then mixed in polypropylene Eppendorf tubes, either with +TBA for sample readings or with -TBA for blank readings. Standards were prepared in tubes using MDA salt, which was mixed with +TBA. All tubes were incubated at 80 ºC for 1 hour while being mixed periodically, then cooled on ice and centrifuged at 12000 g for 15 minutes to pellet out and remove impurities. The supernatant was retained and pipetted into corrosion resistant microplates, which included standards. Absorbance was measured at 450 nm for sugar interference, 532 nm for the estimation of ROS and at 600 nm for interfering phenolics. The calculation method of Zheng *et al.* (2006) for correction of interfering absorbance was used to calculate the amount of MDA present in µmol/l (µmol/l = 6.45 x (A532 - A600) - 0.56 x (A450)).

4.2.7 Total Antioxidants Assay

The assay was performed according to Benzie and Strain (1996) and Wong *et al.* (2006), but with modifications for avocado roots. This assay is known as the ferric reducing ability of plasma (FRAP) and the colour produced by the reduction of complexed ferric ions by total antioxidants, at low pH, is measured at an absorbance of 600 nm. This absorbance can then be compared to the absorbance of a known amount of complexed antioxidant standard, in order to quantify total antioxidants.

Optimisations were performed on sample and standard dilutions for reagent mix ratios, proper blanking technique, optimal absorbance wavelength, prevention of absorbance saturation, which aided the construction of a standard curve (Figure 4.4) using (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX) (Sigma-Aldrich Pty. Ltd., product number 238813).

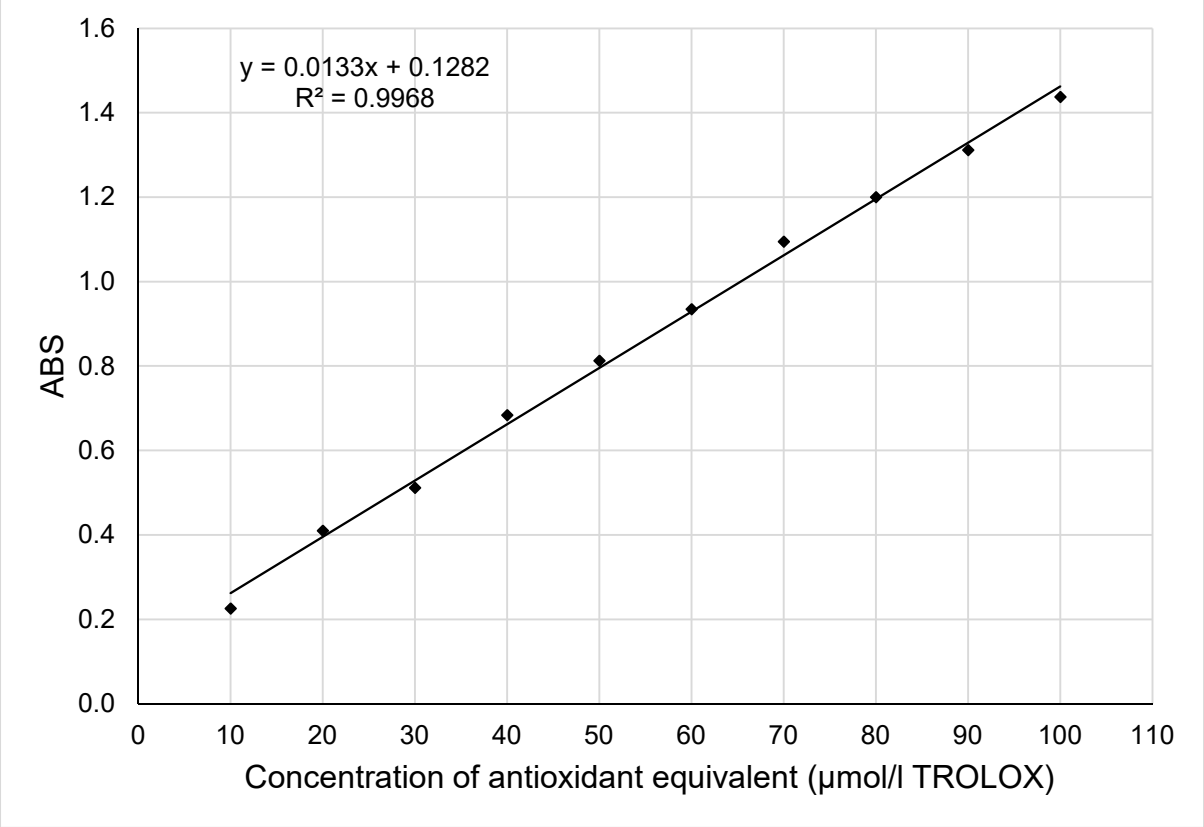

Figure 4.4: Antioxidant standard curve. Absorbance is abbreviated as ABS and total antioxidant content was measured in equivalents of the antioxidant (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX) in µmol/l.

From each sample, 0.1 g was weighed and mixed with 1 ml of 80 % ethanol at 4 ºC, and centrifuged at 14000 g for 10 minutes to extract the antioxidants from solid tissue. The supernatant was retained at 4 ºC and used to prepare sample and blank mixes.

The sample mixes were prepared by the addition of 0.26 ml of 80 % ethanol, 0.27 ml of 0.3 M acetate buffer at pH 3.6, 0.027 ml of 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl and 0.027 ml of 20 mM ferric chloride hexahydrate to the supernatant. The blank mixes contained the same as sample mixes, but ferric chloride hexahydrate was replaced with distilled water to prevent colour complex formation. The antioxidant standard mixes contained the same as the sample mixes, but samples were replaced with TROLOX. All mixes were placed on ice for 20 minutes and then pipetted into a polypropylene microplate for absorbance measurement at 600 nm. Finally, the amount of antioxidant in µmol/l of TROLOX equivalents present in blank corrected samples was determined by a standard curve which was generated by the standards.

4.3 Results

4.3.1 Callose

The ANOVA of callose data (Table 4.1) showed a high significance at the 1 % level for both the overall model and the difference between infected and uninfected rootstocks across an average of time points and rootstock types, which indicated that the data were suitable for further analysis. Independent *t*-tests were used to compare the means, and the results are plotted in scatter plots for visualisation over time (Figure 4.5 and 4.6).

At 6 hpi, all infected rootstocks produced significantly more callose than their controls, and susceptible R0.38 showed the largest difference in means (between infected and control plants), followed by resistant R0.06 and lastly resistant R0.09 (Figure 4.6). At 9 hpi, both infected R0.06 and R0.38 produced significantly more callose than their

controls, and R0.06 showed the largest difference in means, followed by R0.38. However, infected R0.09 did not produce significantly more callose than its control. At 12 hpi, infected R0.09 produced significantly more callose than its control but infected R0.06 and R0.38 did not produce significantly more callose than their controls. The difference in means in R0.09 was the largest in the trial. R0.06 showed a negative difference in means, but as this was quite small (-0.09 µg/ml) and close to zero, it was considered negligible. At 24 hpi, all infected rootstocks produced significantly more callose than their controls, and R0.09 showed the largest difference in means (second highest difference in the trial), followed by R0.38 and lastly R0.06. The overall change in the difference in means over all time points suggested that R0.09 responded earlier to infection than R0.06 and R0.38 in terms of defence callose production. This can be seen in the pattern of the decrease at 12 hpi in R0.06 and R0.38, followed by an increase, was seen earlier in R0.09 at 9 hpi.

Table 4.1: Analysis of variance results from the data of callose produced in response to infection.

Source	**df**	**F**	**Sig.**	**C.V.**
Corrected model	23	14.395	<0.001**	< 1.748
Infection	1	172.849	<0.001**	< 4.001
Error	116			

The full factorial model was applied during analysis. The heading df indicates degrees of freedom, F the F-test value, sig. the significance and C.V. the critical value of F. F statistics investigated were greater than their C.V. Corrected model tested for overall significance of the model and infection for a significant response to infection across an average of time points and rootstock types. Error shows the degrees of freedom for error. Significance at the 1 % level is denoted as **.

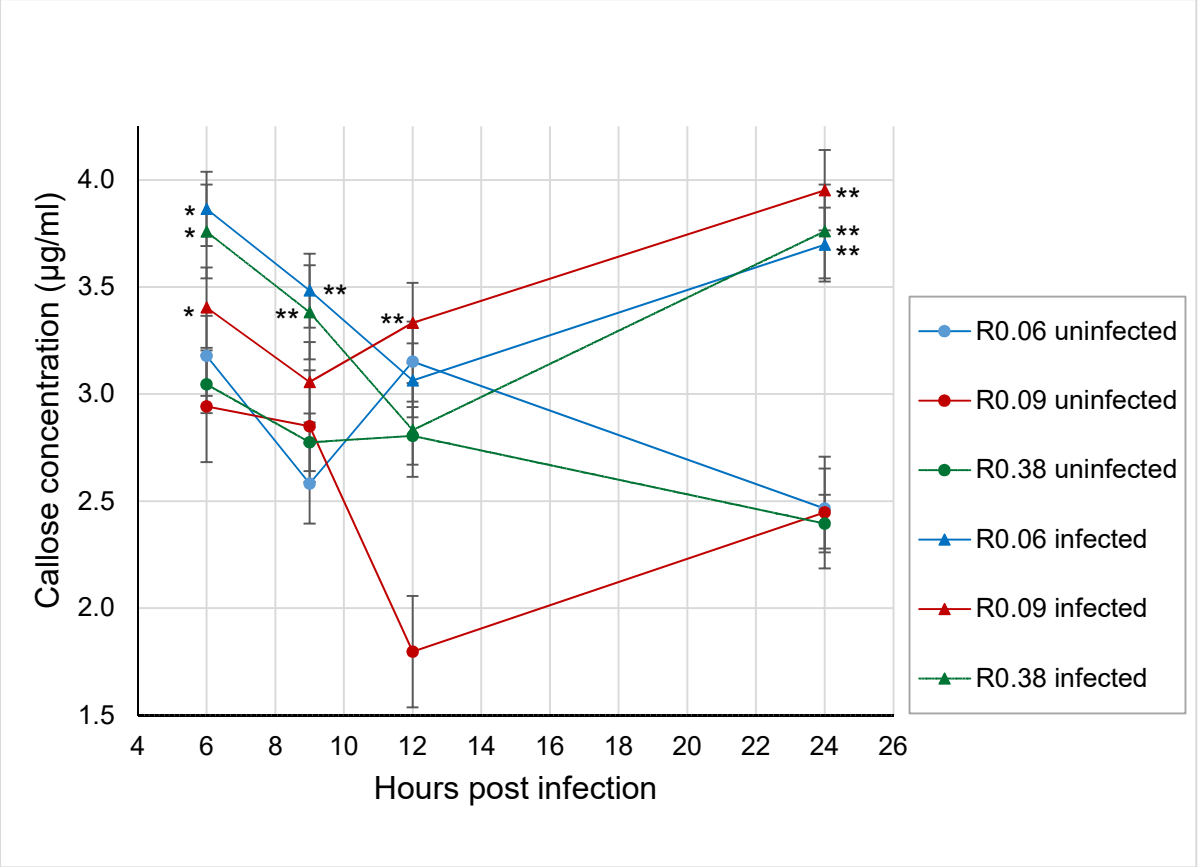

Figure 4.5: Mean callose concentrations in infected and uninfected avocado rootstocks from 6 to 24 hours post infection. Error bars show the standard error. Significant differences between infected rootstocks and their controls (uninfected) at a specific time point are shown as * for the 5 % level and ** for 1 %.

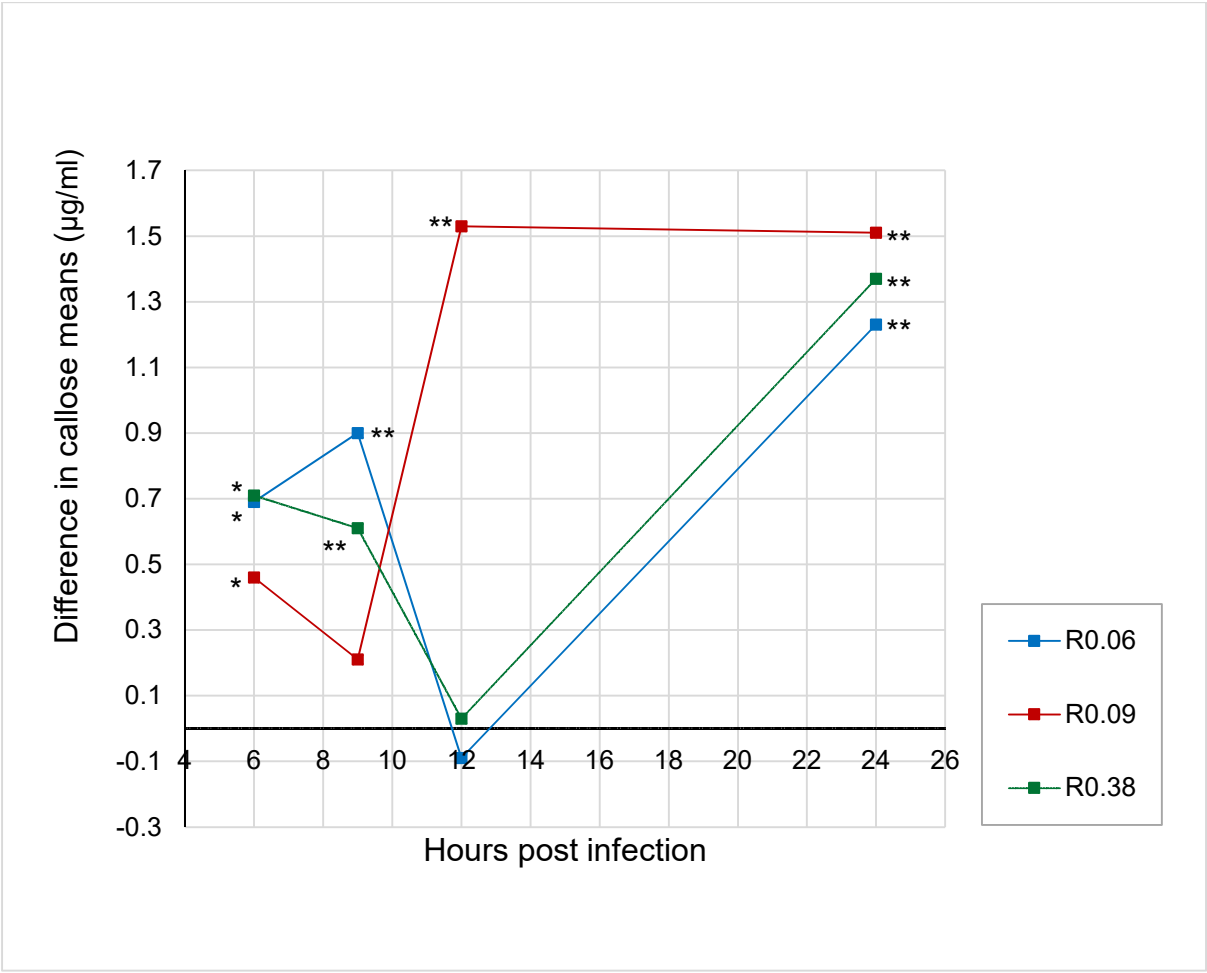

Figure 4.6: Differences between infected and uninfected callose means for rootstocks from 6 to 24 hours post infection. The difference indicates the amount of callose produced only in response to infection. Significant differences are denoted as * at the 5 % level and ** at 1 %.

4.3.2 Lignin

The ANOVA of lignin data (Table 4.2) exhibited a high significance at the 1 % level for the overall model, and a weak significance of the difference between infected and uninfected rootstocks over an average of time points and rootstock types, which suggested that the data were suitable for further analysis. Independent *t*-tests were used to compare the means, and the results are plotted in scatter plots for visualisation over time (Figure 4.7 and 4.8).

At 6, 9 and 12 hpi, all infected rootstocks did not produce significantly more lignin than their controls (Figure 4.7 and 4.8). At 24 hpi, infected R0.09 produced significantly less lignin than its control, infected R0.06 produced marginally less (weakly significant) than its control and infected R0.38 did not produce significantly less than its control. However R0.38 showed a p-value of 0.111, which was close to weak significance. R0.09 showed the largest difference in means at 24 hpi (and in the whole trial), followed by R0.06. The overall change in the difference in lignin means over all time points showed a similar pattern in all rootstocks, with no significant differences over the first three time points and then a significant or noteworthy decrease from 12 to 24 hpi. This suggests that all the rootstocks reacted to infection with similar timing in lignin deposition.

Table 4.2: Analysis of variance results from the data of lignin produced in response to infection.

Source	**df**	**F**	**Sig.**	**C.V.**
Corrected model	23	2.467	0.001**	<1.700
Infection	1	3.362	0.069^{weak}	<4.001
Error	112			

The full factorial model was applied during analysis. The heading df indicates degrees of freedom, F the F-test value, sig. the significance and C.V. the critical value of F. The F statistic for infection was not greater than the C.V. Corrected model tested for overall significance of the model and infection for a significant response to infection across an average of time points and rootstock types. Error shows the degrees of freedom for error. Significance at the 10 % level is denoted as ^{weak} and 1 % as **.

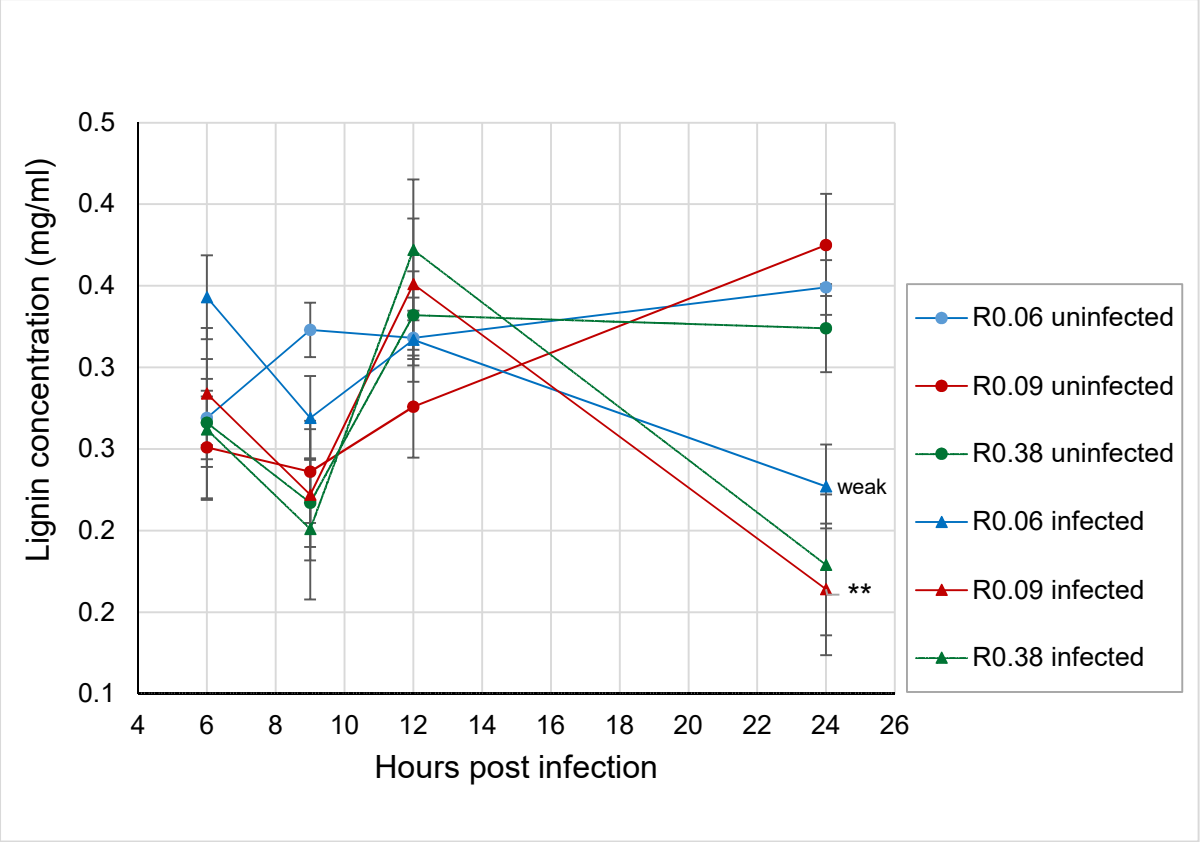

Figure 4.7: Mean lignin concentrations in infected and uninfected avocado rootstocks from 6 to 24 hours post infection. Error bars show the standard error. Significant differences between infected rootstocks and their controls (uninfected) at a specific time point are shown as ^{weak} for the 10 % level and ** for 1 %.

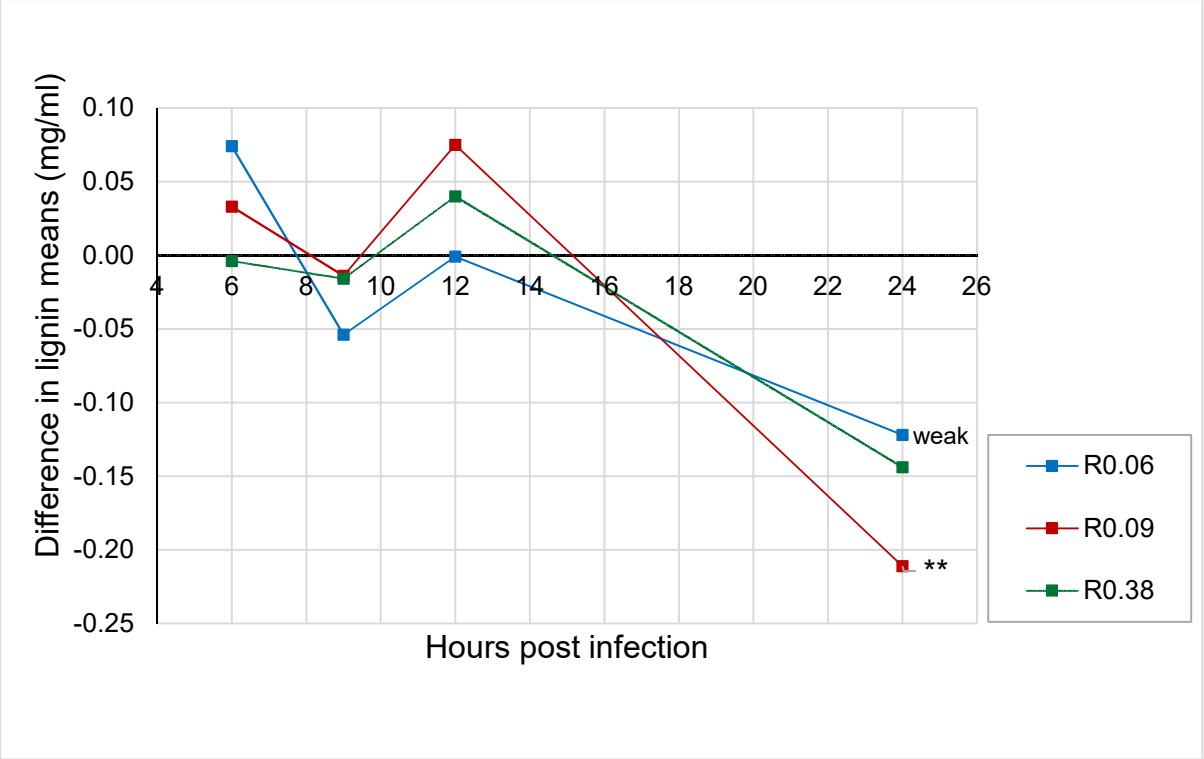

Figure 4.8: Differences between infected and uninfected lignin means for rootstocks from 6 to 24 hours post infection. The difference indicates the amount of lignin produced only in response to infection. Significant differences are denoted as ^{weak} at the 10 % level and ** at 1 %.

4.3.3 Reactive Oxygen Species

Although the ANOVA of ROS data (Table 4.3) showed significance at the 5 % level for the overall model, but not of the difference between infected and uninfected rootstocks across an average of time points and rootstock types, the ROS data were analysed further to provide a comparison for the antioxidant data. Independent *t*-tests were used to compare the means, and the results are plotted in scatter plots for visualisation over time (Figure 4.9 and 4.10).

All infected rootstocks at all time points did not produce significantly less or more ROS compared to their controls (Figure 4.9 and 4.10). It was however noteworthy for the independent *t*-tests that resistant R0.09 showed a p-value of 0.159 at 6 hpi, 0.117 at 12 hpi and resistant R0.06 showed a p-value of 0.156 at 6 hpi. However susceptible R0.38 showed higher p-values at all time points indicating the low probability of any difference between infected rootstocks and controls.

Table 4.3: Analysis of variance results from the data of reactive oxygen species produced in response to infection.

Source	**df**	**F**	**Sig.**	**C.V.**
Corrected model	23	1.840	0.019*	<1.608
Infection	1	0.137	0.712	
Error	111			

The full factorial model was applied during analysis. The heading df indicates degrees of freedom, F the F-test value, sig. the significance and C.V. the critical value of F. The F statistic for infection was not greater than its C.V. Corrected model tested for overall significance of the model and infection for a significant response to infection across an average of time points and rootstock types. Error shows the degrees of freedom for error. Significance at the 5 % level is denoted as *.

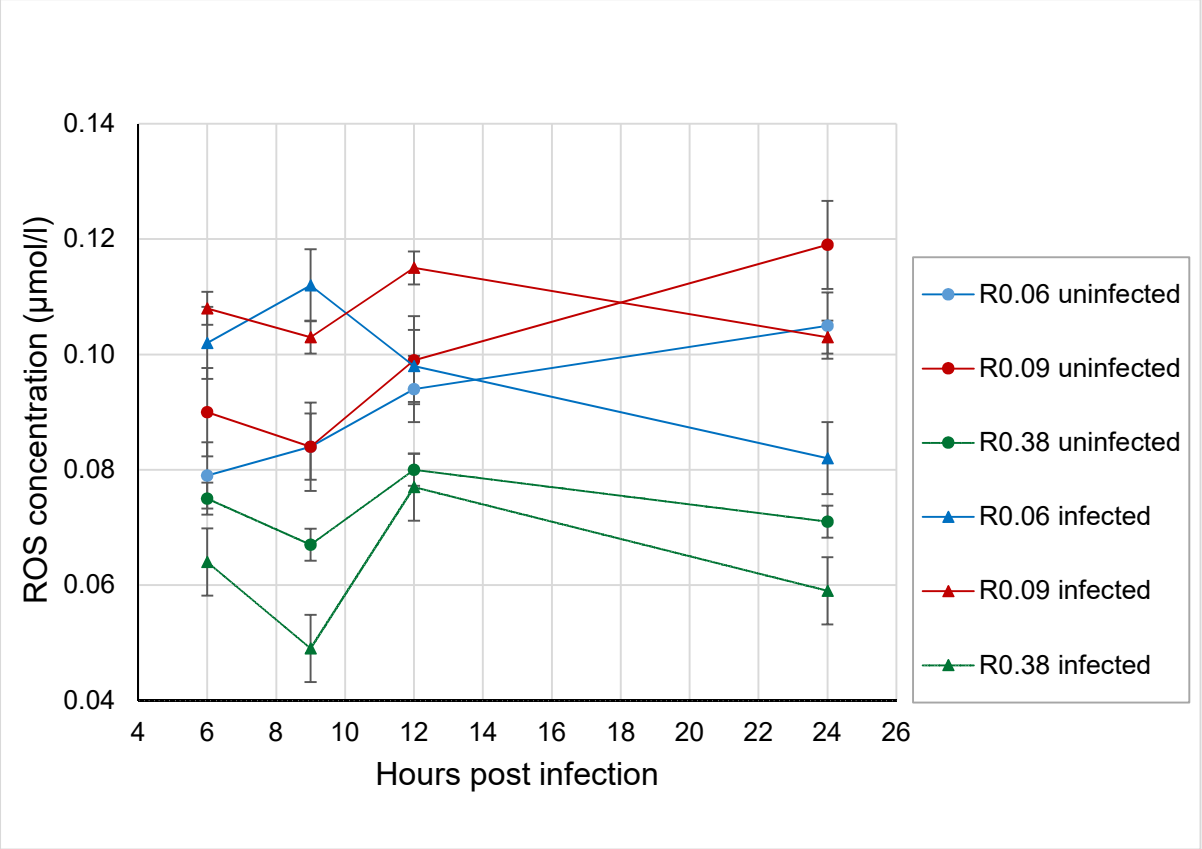

Figure 4.9: Mean reactive oxygen species concentration in infected and uninfected avocado rootstocks from 6 to 24 hours post infection. Error bars show the standard error. There were no significant differences.

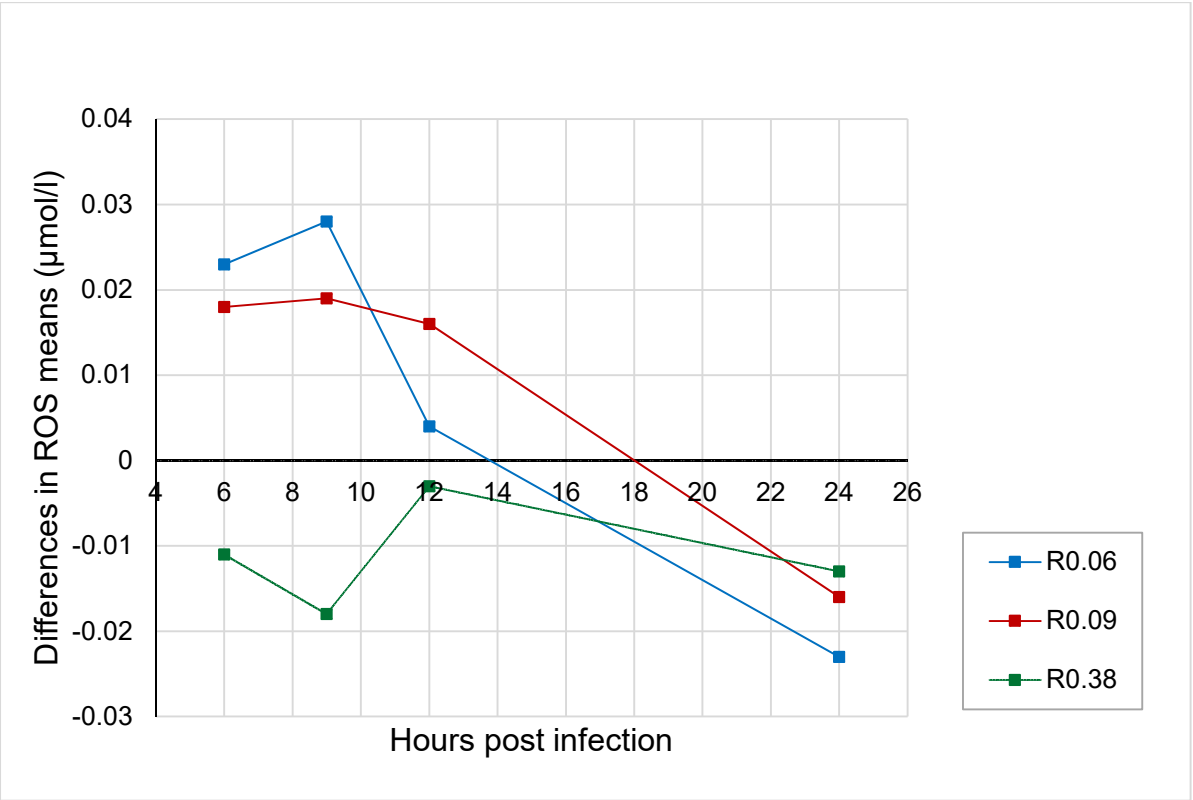

Figure 4.10: Differences between infected and uninfected reactive oxygen species means for rootstocks from 6 to 24 hours post infection. The difference indicates the amount of reactive oxygen species (ROS) produced only in response to infection. There were no significant differences.

4.3.4 Total Antioxidants

The ANOVA of antioxidant data (Table 4.4) exhibited a high significance at the 1 % level for the overall model and a weak significance of the difference between infected and uninfected rootstocks across an average of time points and rootstock types, which suggested that the data were suitable for further analysis. Independent *t*-tests were used to compare the means, and the results are plotted in scatter plots for visualisation over time (Figure 4.11 and 4.12).

At 6 hpi, only infected susceptible R0.38 produced marginally less (weak significance) antioxidants compared to its control (Figure 4.12). At 9 hpi, only infected R0.06 produced significantly less antioxidants than its control. At 12 hpi, only infected R 0.09 produced marginally more (weak significance) antioxidants compared to its control. At 24 hpi, all infected rootstocks did not differ significantly from their controls. In the overall change in antioxidant production, infected R0.38 might have reacted earlier than infected R0.06 as compared to their controls, because it showed an earlier significant decrease.

Table 4.4: Analysis of variance results from the data of total antioxidants produced in response to infection.

Source	**df**	**F**	**Sig.**	**C.V.**
Corrected model	23	3.392	<0.001**	<1.700
Infection	1	3.688	0.057^{weak}	<4.001
Error	109			

The full factorial model was applied during analysis. The heading df indicates degrees of freedom, F the F-test value, sig. the significance and C.V. the critical value of F. The F value of infection was less than its C.V. Corrected model tested for overall significance of the model and infection for a significant response to infection across an average of time points and rootstock types. Error shows the degrees of freedom for error. Significance at the 10 % level is denoted as ^{weak} and at 1 % as **.

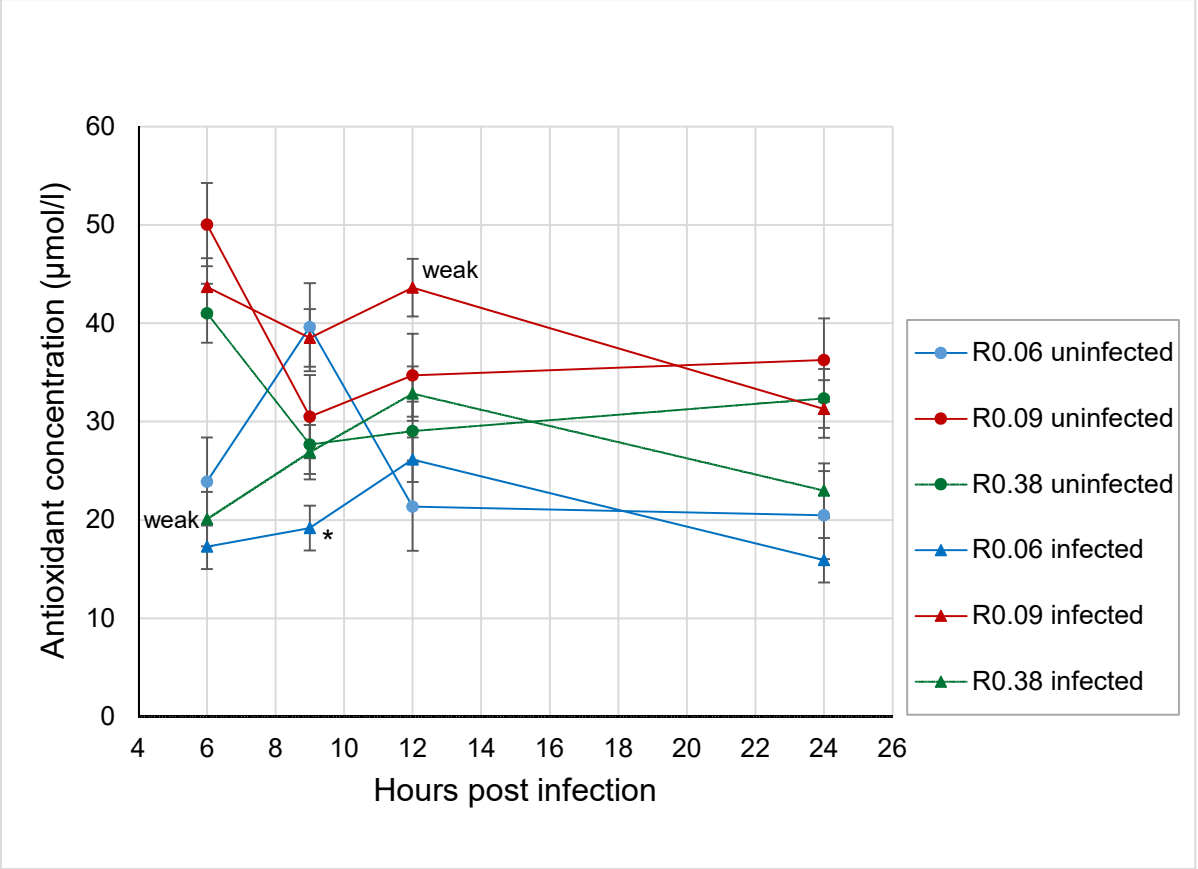

Figure 4.11: Mean antioxidant concentrations in infected and uninfected avocado rootstocks from 6 to 24 hours post infection. Error bars show the standard error. Significant differences between infected rootstocks and their controls (uninfected) at a specific time point are shown as, ^{weak} for significance at the 10 % level and * for 5 %.

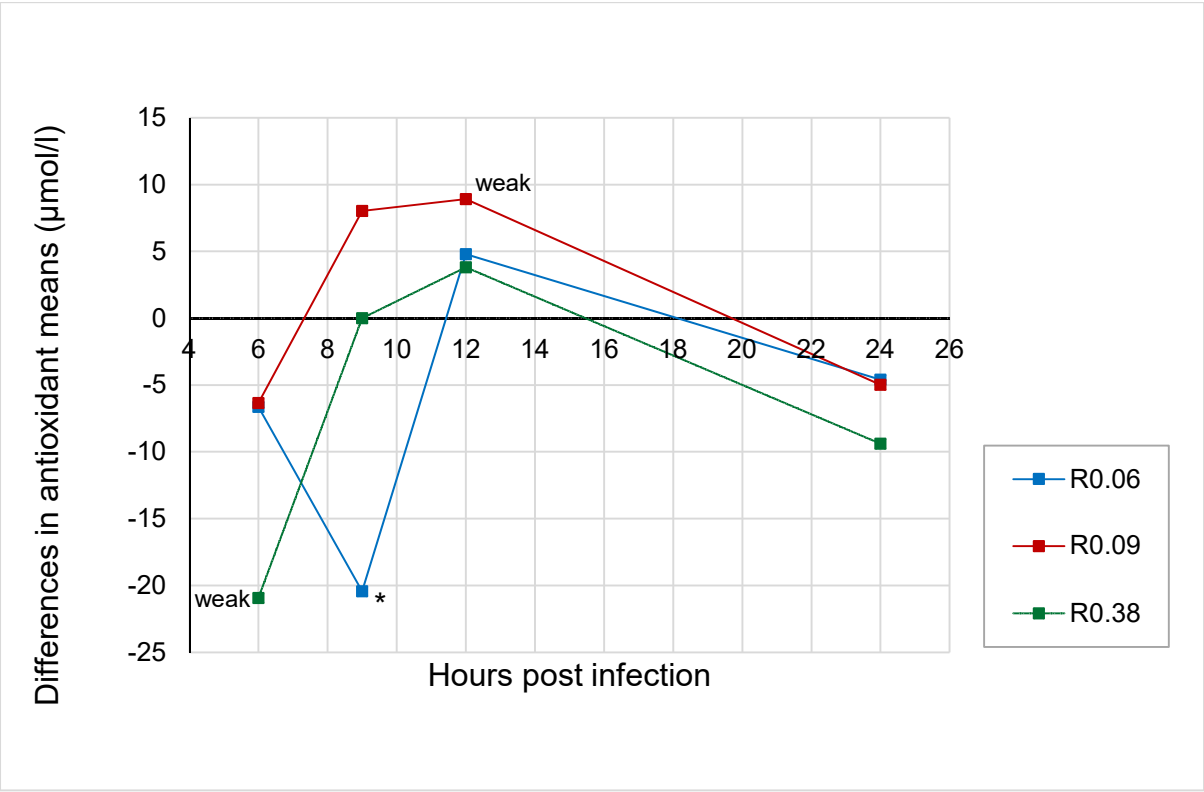

Figure 4.12: Differences between infected and uninfected antioxidant means for rootstocks from 6 to 24 hours post infection. The difference indicates the amount of antioxidants produced only in response to infection. Significant differences are denoted as ^{weak} at the 10 % level and * at 5 %.

4.4 Discussion

This study used quantitative assays to determine the concentration of callose, lignin, ROS and total antioxidants produced in resistant R0.06, resistant R0.09 and susceptible R0.38 avocado rootstocks, in response to infection with *Pc*. The data collected was analysed using ANOVA, and differences in the means between infected and control plants were tested using independent *t*-tests.

It is first necessary to consider the effect of the infection strategy of *Pc*, as a known hemibiotroph, on the avocado defence response (Hardham and Blackman, 2018). The

pathogen initially acts as a biotroph, which does not kill cells but establishes haustoria against cell membranes to absorb nutrients (Agrios, 2005, Hardham, 2005, Oßwald *et al.*, 2014, Hardham and Blackman, 2018). It then undergoes a biotrophic to necrotrophic switch (BNS) at approximately 12 hpi in avocado (Backer *et al.*, 2015), and becomes a necrotroph which destroys plant cells and feeds on their contents (Agrios, 2005, Oßwald *et al.*, 2014, Hardham and Blackman, 2018). As the necrotrophic phase of pathogens is more damaging than the biotrophic phase (Vargas *et al.*, 2012), avocado rootstocks possibly exhibit a stronger defence response after BNS.

Callose is a versatile polymer in plants (Chen and Kim, 2009), that strengthens cell walls to stop pathogens from reaching cells, and is thought to be associated with resistance to *Pc* (Jacobs *et al.*, 2003, Piršelová and Matušíková, 2013, Ellinger and Voigt, 2014, van den Berg *et al.*, 2018). Hence it was hypothesised that resistant rootstocks would produce earlier or more callose than susceptible rootstocks in response to infection. However, neither the earliness nor the amount of callose produced could be linked to resistance because at 6 hpi, the infected susceptible R0.38 produced the most callose compared to its control, which was more than seen in the resistant rootstocks. The higher amount of callose produced at 6 and 9 hpi in infected R0.38 and R0.06, compared to their controls, suggests that they could have better early callose defence as compared to R0.09. This is because the pathogen would have to penetrate or bypass more callose before reaching cells for nutrients. However, infected R0.09 produced significantly more callose than its control at the proposed BNS and at 24 hpi, and these observations were respectively the highest and second highest callose readings in the whole trial. This significant response of

R0.09 at the proposed BNS, which was absent in the other rootstocks, may limit *Pc*'s access to cells during the more destructive necrotrophic phase and could account for the documented resistance of R0.09. Unexpectedly, infected susceptible R0.38 and infected resistant R0.06 both produced similar amounts of callose across time points in the trial, as compared to their controls. This suggests that R0.06 might not rely on callose for its resistance to *Pc*, or that perhaps that R0.38 has vulnerabilities that *Pc* can exploit, despite its callose deposition, which makes it susceptible. For example, R0.06 could possibly be able to detect effectors produced by *Pc* that R0.38 cannot; or R0.06 may produce other antimicrobial compounds not produced by R0.38, which may account for the documented resistance of R0.06. However, this would require further study.

Although no other quantitative callose studies in *Pc* infected avocado rootstocks are available, the present study agreed with a non-quantitative microscopy study by van den Berg *et al.* (2018), which also showed an early increased callose deposition in R0.06 at 6 hpi in response to infection. However, while acknowledging that R0.38 and R0.12 are both susceptible but differ as rootstocks, R0.12 showed no significant increase in callose production in the first 24 hpi, while R0.38 did in the present study. This indicates that R0.12 may be vulnerable to *Pc* due to its lack of early callose deposition but R0.38 is probably susceptible due to factors other than early callose deposition. These factors may include for example, the failure to detect *Pc* effectors associated with virulence, but this requires further investigation.

Lignin is used to bolster plant cell walls against pathogen entry and to seal off plant cells during the hypersensitive response (HR) in order to prevent additional pathogen

growth (Moerschbacher *et al.*, 1990, Nicholson and Hammerschmidt, 1992). Lignin deposition in *Pc*-infected avocado rootstocks has been associated with susceptibility (van den Berg *et al.*, 2018). Hence it was hypothesised that susceptible R0.38 would produce earlier or larger amounts of lignin in response to infection, compared to the resistant rootstocks. However, this could not be proven, as no significant differences in lignin deposition were observed at 6, 9 and 12 hpi, between infected rootstocks and their controls. Furthermore at 24 hpi, infected R0.09 produced significantly less lignin than its control (largest difference in the trial), infected R0.06 produced marginally less (weak significance) than its control, and infected R0.38 produced a noteworthy negative difference (p-value of 0.111) compared to its control. These decreases in deposited lignin are probably due to dephenolisation by *Pc* (Casares *et al.*, 1986), as lignin cannot be removed enzymatically by plants (Srivastava, 2002). This could suggest that lignin may not be as effective as callose as a defence polymer if it can be removed by *Pc*. It is unlikely that *Pc* produces glucanase enzymes to hydrolyse the β-glucan callose in plants because *Pc* contains it own β-glucan linkages in hyphae that are susceptible to hydrolysis (Aronson *et al.*, 1967, van den Berg *et al.* 2018). In further support of this theory, *Pc* produces glucanase inhibiting proteins to protect itself from glucanase damage (York *et al*., 2004).

Although no other quantitative studies using the acetyl bromide method in avocado rootstocks are available, a qualitative microscopy study by van den Berg *et al.* (2018) found no significant increase in lignin deposition in *Pc* infected R0.06 rootstocks, which agrees with R0.06 in the present study. While considering that R0.38 and R0.12 are both susceptible but differ as types; R0.12 showed an increase in lignin production in response to *Pc* infection at 6 hpi onwards, whereas R0.38 in the present study did not.

This suggests that the rootstocks differ in the mechanism of their susceptibility, which may involve lignin deposition in R0.12. A proteomic study on a highly tolerant *Pc* infected rootstock ('Martin Grande') showed the increased expression of proteins associated with lignin biosynthesis at 12 hpi (Acosta-Muñiz *et al.*, 2012), suggesting that lignin is associated with tolerance to *Pc* in this rootstock. Though tolerance reduces or offsets the negative effects of the pathogen on plant fitness, whereas resistance deters pathogen growth and infection (Horns and Hood, 2012, Pagán and García-Arenal, 2018), there were no comparable significant increases at 12 hpi in the present study. A study conducted by García-Pineda *et al.* (2010) quantified lignin production on a *Pc* infected susceptible rootstock using the thioglycolic method and found no significant changes overall, but as time points of 0, 2 and 4 days post infection were not included in the present study, relevant comparisons are not possible.

Reactive oxygen species are produced in plants as a systemic stress signal in response to infection (Baxter *et al.*, 2014, Gilroy *et al.*, 2014). Therefore ROS were hypothesised to be produced earlier or in greater amounts in resistant rather than susceptible avocado rootstocks in response to *Pc* infection. However there were no significant differences between infected rootstocks and their controls, and hence the hypothesis could not be tested. Even so, the independent *t*-tests showed that resistant R0.06 at 6 hpi and resistant R0.09 at 6 hpi and 12 hpi had comparatively lower p-values as compared to susceptible R0.38 which showed higher p-values at all time points. This indicates a higher probability of differences between infected resistant rootstocks and their controls as compared to infected R0.38 and its controls, but this requires further investigation. The lack of significant differences may be explained by the potential low sensitivity of the assay, which measured the degree of lipid

peroxidation to estimate ROS production, instead of directly measuring ROS using probes (Hodges *et al.*, 1999, Zheng *et al.*, 2006, Held, 2010, Winterbourn, 2014). It could also be due to the instability of ROS, which makes them difficult to study quantitatively (Sies, 1993, D'autréaux and Toledano, 2007). A study by García-Pineda *et al.* (2010) also used the absorbance of MDA to quantify ROS produced in response to *Pc* infection in a susceptible rootstock, but significant differences were seen at 2 and 4 days post infection, which were considerably later time points than the present study, hence not allowing for suitable comparison.

Antioxidants control ROS levels (Blokhina *et al.*, 2003), and hence it was hypothesised that if resistant avocado rootstocks produce more ROS than susceptible rootstocks in response to infection, then they would also be able to produce more antioxidants to control increased ROS levels. However, no significant differences in ROS production were observed, so it was not possible to compare antioxidant and ROS levels to test the hypothesis. Nonetheless, as antioxidant levels increase in plants to scavenge ROS produced in response to infection (Mittler *et al.*, 2004), the weakly significant antioxidant increase at 12 hpi in infected R0.09 compared to its control, may have been produced to scavenge earlier signal ROS that were possibly produced before 6 hpi. This could imply that infected R0.09 reacted earlier than other rootstocks, which would associate antioxidant production with resistance. Furthermore, as antioxidant activity is supressed during defence ROS production in response to infection (Mittler, 2002), the marginal antioxidant decrease (weakly significant) in infected susceptible R0.38 at 6 hpi compared to its control and the significant decrease in infected resistant R0.06 at 9 hpi compared to its control, could have allowed for signal ROS production. However, further ROS studies would be required to verify this.

Two quantitative antioxidant studies performed on *Pc* infected rootstocks are noted as follows. Firstly, van den Berg *et al.* (2018) observed decreased catalase activity for the first 12 hpi, followed by an increase in superoxide dismutase (SOD) activity at 12 hpi and then an increase in catalase activity at 24 hpi in resistant R0.06. However susceptible R0.12 showed decreased catalase activity for the first 12 hpi but only showed an increase in SOD activity at 48 hpi. This suggested that R0.06 responded to *Pc* with earlier ROS production than R0.12, and the increase in catalase activity at 24 hpi could have scavenged excess ROS in R0.06. Before comparing, the following admonitory differences are noted. The present study assayed total antioxidants, whereas the past study did not include all antioxidants. In addition, the present study used a control at every time point for each rootstock, whereas the past study only used a zero hour control. This would allow for better correction for the response of control plants over time in the present study, which increases experimental accuracy. Lastly, although both R0.38 and R0.12 are susceptible, they differ phenotypically from eachother, and both R0.09 and R0.06 are resistant, but also differ phenotypically from eachother. In comparing the studies, the decrease in catalase activity in the first 12 hpi in both rootstocks could be the reason for the decrease in total antioxidants in infected R0.38 and R0.06 at 6 hpi and 9 hpi respectively, compared to their controls, which may have allowed for signal ROS propagation. In addition, the increase in SOD activity at 12 hpi in R0.06 could be the reason for the increase in total antioxidants at 12 hpi in R0.09, and the positive differences in means in R0.06 and R0.38 at 12hpi. This may suggest that signal ROS were produced in defence earlier than 12 hpi, as SOD converts superoxide produced by respiratory burst oxidase homolog enzymes (RBOHs) to hydrogen peroxide (Gilroy *et al.*, 2014). Secondly, a proteomic study on a

highly tolerant *Pc* infected rootstock ('Martin Grande') showed increased expression of proteins associated with glutathione antioxidant activity at 12 hpi (Acosta-Muñiz *et al.*, 2012). Bearing in mind that the rootstocks used in the present study are resistant and not tolerant, and that the present study represents total antioxidants, perhaps the increase at 12 hpi in R0.09 in the present study might be due to an increase in glutathione. This may be of future interest as glutathione is found in the apoplast and could potentially scavenge signal ROS (Mittler, 2002, Podgórska *et al.*, 2017).

A holistic interpretation of all observations is as follows. As inducible plant defence responses require pathogen detection and a signal cascade to cause the expression of defence genes (Jones and Dangl, 2006, Eckardt, 2017), the increased callose deposition in response to infection at 6 hpi in all rootstocks must have resulted from the successful detection of *Pc* pathogen associated molecular patterns (PAMPs) or effectors. Some *Pc* effectors and PAMPs are known to be produced by *Pc* in the first 24 hpi (Hardham and Blackman, 2018), which was the duration of the trial in this study, thus suggesting that effectors would have been present. The sizable callose response by infected R0.09 at the assumed BNS, thought to be linked to its documented resistance, must have resulted from the successful detection of the assumed BNS or at least a change in the pathogen virulence, possibly involving the secretion of *Pc* effectors such as cell wall degrading enzymes. Although callose deposition can result from ROS signalling (Daudi et al., 2012), comparison could not be made as no significant changes in ROS as a result of infection were detected. However, the absence of ROS did correspond with the lack of significant increases in lignin deposition in response to infection because apoplastic ROS produced by RBOHs are required for lignin polymerisation (Gilroy *et al.*, 2014, Barros *et al.*, 2015).

Nevertheless, the significant changes in antioxidant production in response to infection alluded to possible changes in ROS activity, which may have been too small to be detected in the ROS assay, may still be of interest to further investigation with regard to defence signalling.

Unfortunately, experimental limitations in this study could have affected the results, and are explained as follows. Firstly, the β-glucan linkages present in *Pc* hyphae may have increased the fluorescence detected from infected samples of the callose assay (Aronson *et al.*, 1967). Despite this possibility, a similar assay was successfully used to quantify callose in *Pc* infected resistant *Lomandra longifolia*, and showed significant callose increases from 48 hpi onwards (Islam *et al.*, 2017), suggesting that the assay can detect changes in callose. In addition, the amount of *Pc* in root samples would increase over time, as a result of colonisation, thereby increasing the bias. However, as the present study only proceeded for 24 hpi, it was at lower risk of bias as compared to the study by Islam *et al.* (2017), where callose was quantified until 168 hpi. Secondly, as quantitative analysis of ROS is difficult, the investigation could have benefited from the qualitative use of microscopy and fluorescent ROS probes or dyes (Winterbourn, 2014). However, ROS are unstable and have a short half-life (Sies, 1993, D'autréaux and Toledano, 2007), the use of live tissue and close observation after infection would be required, and this was not possible with the scale of trial used in the present study.

Future studies should be centred on verifying the timing of BNS to understand when *Pc* becomes more damaging to avocado rootstocks. The identification of the sizable callose deposition in infected R0.09 at the assumed BNS should be used to help find

resistance markers, perhaps involving gene knock-outs or inhibition of callose synthase to verify causality. The use of quantitative real-time PCR to monitor *Pc* pathogen load is not recommended for early defence studies within the first 24 hpi as relatively little *Pc* DNA is detected due to the short time span (Engelbrecht et al., 2013). Earlier time points should be examined to find the timing of the earliest defence response, as significant differences in callose means between infected rootstocks and their controls were already evident by 6 hpi. In addition, the overall similar pattern of change in the difference in callose means between the susceptible R0.38 and resistant R0.06, which differed from resistant R0.09 (Figure 4.6), should be examined to reveal why the two resistant rootstocks responded differently or why the resistant R0.06 and susceptible R0.38 rootstock responded similarly in response to *Pc* infection. The efficacy of *Pc* to degrade lignin in rootstocks can be tested, but lignin appears not to be an effective defence polymer against *Pc* infection. Lastly, more sensitive ROS assays, or perhaps those specifically targeting apoplastic RBOHs, should be used to provide a ROS reference point for the activity of antioxidants in response to *Pc* infection and to reveal the role of signalling ROS in defence against *Pc*.

4.5 Conclusion

Callose production in rootstocks before the proposed BNS was associated more with the infected susceptible R0.38, as it produced the most callose at the earliest time point of 6 hpi compared to its control; although the infected resistant R0.06 followed closely in amount compared to its control. Overall, however, callose production was possibly associated with resistance to *Pc*, as the infected resistant R0.09 responded to the proposed BNS by the deposition of a high amount of callose, as compared to its control, which was not observed in the other rootstocks and may have been an

effective barrier against the destructive necrotrophic growth of *Pc*. This information is valuable as a potential biochemical resistance marker and should be used in further research. Additionally, the overall pattern of change in the differences in callose means was similar between susceptible R0.38 and resistant R0.06, but not resistant R0.09 (Figure 4.6). This suggests that the role of callose in the mechanism of resistance against *Pc* differs between the resistant rootstocks, and should be investigated further. Lignin production could not be associated with susceptibility to *Pc* in this study, as there were no significant increases in lignin production between infected rootstocks and their controls. However, there was an unexpected significant decrease in lignin in infected resistant rootstocks at 24 hpi compared to their controls. This was possibly due to the degradation of lignin by *Pc*, and should be further investigated to determine the efficacy of lignin as a defence polymer, but lignin seems unimportant in the search for resistance markers thus far. The production of ROS was thought to be associated with resistance, but this could not be established as no significant differences were observed in the present study. This could possibly have been due either to the unstable nature of ROS, or to the likely insensitivity of the indirect means of ROS quantification in the assay. Hence future studies should include assays that specifically target apoplastic RBOHs or use direct quantification, or live tissue microscopy involving ROS probes. As no significant changes were observed in ROS production, it was not possible to use ROS as a reference point for antioxidant production, which was hypothesised to be associated with resistance. However, if infected R0.09 had produced ROS in defence before 12 hpi the weak significant increase in antioxidants at 12 hpi, compared to its control, could have been to attenuate signalling ROS and hence antioxidants could have been associated with resistance in R0.09. However, this would require further studies for verification. Furthermore, the significant

antioxidant decreases in infected R0.38, and R0.06 at 6 and 9 hpi respectively, compared to their controls, may have been to allow for signal ROS production, but this too would require further research.

4.6 References

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Chapter 5

General Discussion

The principal challenge to the avocado industry is the production loss as a result of Phytophthora root rot (PRR), which is caused by the oomycete pathogen *Phytophthora cinnamomi* Rands (*Pc*) (Ploetz, 2003, Wolstenholme, 2010). This oomycete infects and damages avocado tree root systems (Hardham, 2005, Hardham and Blackman, 2018), thus causing yield reduction and possible death in susceptible trees (Zentmyer, 1953, Eskalen, 2008, Oßwald *et al.*, 2014). *Pc* is difficult to eliminate once established, but PRR can be effectively managed using a holistic approach. One promising management aspect is the use of *Pc*-resistant rootstocks, which may provide a lasting solution (Pegg, 1978, Menge *et al.*, 2002, Wolstenholme, 2010). The lengthy breeding and selection process of these rootstocks could be accelerated by marker assisted selection, but this requires prior marker knowledge (Ben-Ya'acov and Michelson, 1995). Hence the purpose of this dissertation was to investigate the defence response of avocado rootstocks to infection with *Pc* in order to assist future identification of defence markers. The defences investigated were the cell wall thickening by callose and lignin, the phenolic accumulation in cells, the production of tyloses and gum in xylem vessels, the production of reactive oxygen species (ROS) and the production of antioxidants to control ROS levels.

The glucose polymer callose is used to reinforce cell walls against pathogen entry in plants (Chen and Kim, 2009, Piršelová and Matušíková, 2013). It was discussed in Section 2.3.3 of Chapter 2, and it has been associated with the resistance of plants to pathogens (Enkerli *et al.,* 1997, Eggert *et al.,* 2014), to *Pc* (Hinch and Clarke, 1982, Cahill and Weste, 1983, Allardyce, 2011) and in the resistance of avocado rootstocks to *Pc* (van den Berg *et al.* (2018a). Hence callose was hypothesised to be produced

earlier or in greater quantities in resistant rather than in susceptible rootstocks when infected with *Pc*.

Although the microscopy study of callose deposition proved inconclusive, the callose assay results did not support the hypothesis because the infected susceptible R0.38 produced more callose, compared to its control, than was produced by the infected resistant rootstocks, compared to their controls at the earliest time point of 6 hpi. The early callose deposition in R0.06 agreed with the study by van den Berg *et al.* (2018a), but it was unexpected that the susceptible R0.38 would deposit so much early callose. Nevertheless, infected R0.09 deposited the highest amount of callose in the whole trial at 12 hpi as compared to its control, whereas the other infected rootstocks did not produce significant callose increases at this time. This observation was important as *Pc* is a hemibiotroph (Hardham and Blackman, 2018), and is thought to undergo a biotrophic to necrotrophic switch (BNS) at around 12 hpi in avocado (Backer *et al.*, 2015). The necrotrophic phase in plants is much more destructive than the stealthy biotrophic phase (Vargas *et al.*, 2012) and hence R0.09 may have successfully detected BNS, and increased callose deposition to stop or slow the pathogen from damaging cells. In conclusion, this observation may be responsible for the documented resistance of R0.09 and is potentially a biochemical resistance marker.

The phenolic biopolymer lignin is used in plant defence, either to bolster cell walls against intruding pathogens or to seal off cells during the hypersensitive response (Moerschbacher *et al.*, 1990, Nicholson and Hammerschmidt, 1992). It was discussed in Section 2.3.6 of Chapter 2, and has been associated with resistance of plants to pathogens (Gayoso *et al.*, 2010, Xu *et al.*, 2011), and more with resistance than

susceptibility of plants to *Pc* (Cahill *et al.*, 1989). However, the role of lignin in the response of avocado rootstocks to *Pc* has been unclear (García-Pineda *et al.*, 2010, Acosta-Muñiz *et al.*, 2012, Reeksting *et al.*, 2014, van den Berg *et al.*, 2018b), but van den Berg *et al.* (2018a) found that lignin deposition was associated with a susceptible R0.12 rootstock, whereas a resistant R0.06 roostock produced callose instead. Hence, it was hypothesised that the susceptible R0.38 rootstock would produce more or earlier lignin deposition than the resistant rootstocks in the present study.

However, this could not be confirmed as the microscopy study of lignin deposition was inconclusive and the quantitative lignin assay did not show any significant increases in lignin deposition in response to infection in any rootstocks. Hence the amount of lignin deposited in response to infection did not seem to be associated with resistance or susceptibility to *Pc*. Nevertheless, at 24 hpi there were significant quantitative decreases in the amount of lignin present in infected resistant rootstocks compared to their controls and a noteworthy decrease (p-value of 0.111) in R0.38 compared to its control. These decreases could be the reason for the lack of lignin observations at 24 hpi in the microscopy study, and may have been caused by the ability of *Pc* to dephenolise lignin (Casares *et al.*,1986). In conclusion, this observation suggests that lignin may not be valuable to research in aid of resistance marker identification in avocado rootstocks.

Reactive oxygen species are produced in plants by respiratory burst oxidase homolog enzymes (RBOHs) as a systemic stress signal in response to pathogens (Apel and Hirt, 2004, Suzuki *et al.*, 2011, Baxter *et al.*, 2014, Gilroy *et al.*, 2014). They were discussed in Section 2.3.4 of Chapter 2, and have been associated with the resistance

of plants to pathogens as well as *Pc* (Rookes *et al.*, 2008, Chaouch *et al.*, 2012, Allardyce *et al.*, 2013). In studies of *Pc* infected avocado rootstocks, early ROS production has been related to resistance (García-Pineda *et al.*, 2010, van den Berg *et al.*, 2018b). Hence for the present study it was hypothesised that ROS production in response to infection would be seen earlier or in greater quantities in the resistant rootstocks as compared to the susceptible rootstock.

However, this could not be confirmed as there were no significant differences in ROS production in response to infection in all rootstocks. The lack of significant observations could have been due to the chemical instability of ROS (Sies, 1993, D'autréaux and Toledano, 2007), or to the potentially less sensitive method of indirect ROS measurement by lipid peroxidation, which may be improved on in future studies by the use of ROS probes with live tissue (Hodges *et al.*, 1999, Zheng *et al.*, 2006, Held, 2010, Winterbourn, 2014).

Antioxidants are produced in plants to control levels of ROS (Blokhina *et al.*, 2003), and can be increased to scavenge ROS (Mittler *et al.*, 2004), or decreased to allow ROS signalling (Mittler, 2002). They were discussed in Section 2.3.4 of Chapter 2. In studies of avocado infected with *Pc*, the production of antioxidants or associated proteins and transcripts, has been associated with resistance or tolerance to *Pc* (Acosta-Muñiz *et al.*, 2012, van den Berg *et al.*, 2018a, van den Berg *et al.*, 2018b). Hence in the present study it was hypothesised that if resistant rootstocks produced more ROS in response to infection, then they would also produce more antioxidants to scavenge the ROS after it has fulfilled its defence function.

Unfortunately, due to the lack of significant changes in ROS concentration, it was not possible to use ROS production as a benchmark to understand resulting changes in antioxidant production. However, there were significant changes in antioxidants in response to infection, which suggested there could have been changes in ROS production that were not detected by the ROS assay. Firstly, significant decreases in antioxidant production were observed in R0.38 at 6 hpi and R0.06 at 9 hpi, may have been produced to encourage defence ROS production, but further investigation is required. Secondly there was a significant increase in antioxidants in R0.09 at 12 hpi, may have been produced to lower ROS levels after an earlier ROS response to infection which could be associated with resistance or the occurrence of BNS, but this requires further study.

Phenolics are used in plants as antimicrobial compounds or to strengthen cell walls in defence against pathogens (Taiz and Zeiger, 2010). They were discussed in Section 2.3.5 of Chapter 2. The production of phenolics has been associated with plant resistance to *Pc* (Cahill *et al.,* 1993, Cahill *et al*., 1989). Furthermore, in *Pc* infected avocado rootstocks, the production of phenolics or associated transcripts has mostly been associated with resistance (Phillips *et al.*, 1987, Engelbrecht and van den Berg, 2013, van den Berg *et al.*, 2018b), except for a single study by van den Berg *et al.* (2018a). Hence the early or increased cytoplasmic accumulation of phenolics in response to infection was thought to be associated with resistance in the present study.

However, this was disproved as the microscopy study showed an early and abundant cortical accumulation of phenolics at 6 hpi in susceptible R0.38, which was not present

in the resistant rootstocks. This agrees with the study by van den Berg *et al.* (2018a), who used a quantitative assay and found higher phenolic accumulation in a susceptible R0.12 rootstock as compared to a resistant R0.06 rootstock. These phenolics may include tannins as suggested by Phillips *et al.* (1987), and are probably antimicrobial in nature as they were observed in abundance in the cytoplasm of cells. Despite their abundance, they were clearly unable to stop *Pc* from reaching the stele at 6 hpi. In conclusion, the production of phenolics is not involved in resistance to *Pc* and would not be helpful in finding resistance markers for avocado rootstocks.

Tyloses are formed by the growth of adjacent cells into xylem vessels (Zimmermann, 1979), and can be produced in response to pathogens in plants (De Micco *et al.*, 2016). Tyloses are associated with gums, which are polysaccharide and pectin based cell secretions that can be preceded by gels (Agrios, 2005, Rioux *et al.*, 1998). Tyloses and gums were discussed in Section 2.3.7 of Chapter 2. The production of tyloses has been associated with susceptibility of trees to pathogens (Parke *et al.*, 2009, Inch *et al.*, 2012), but in *Pc* infected avocado rootstocks, their role has been ambiguous (Phillips *et al.*, 1987, van den Berg *et al.*, 2018a). The production of tyloses and gums in response to infection was hypothesised to be associated with susceptibility in the present study.

However, the production of tyloses could not be associated with resistance or susceptibility as they were seen in both resistant R0.09 and susceptible R0.38 at the same time point of 12 hpi and hence this requires further study, which may be aided by the use of longitudinal root sections and confocal microscopy. The early presence of gum deposits in xylem vessels in response to infection was not associated with

resistance, but possibly with susceptibility as they were first observed in the susceptible R0.38 at 6 hpi. In conclusion, the formation of gums is unimportant to the search for resistance markers in avocado rootstocks. No other studies on avocado with *Pc* have observed the formation of gums or gels to date.

In concert, the pathogen and host may have interacted as follows. *Pc* is known to produce effectors and pathogen associated molecular patterns (PAMPs) in the first 24 hpi (Hardham and Blackman, 2018), and the successful detection of these is necessary for the inducible defence responses of plant cells (Jones and Dangl, 2006, Doughari, 2015, Eckardt, 2017). Detection by receptors in the cell membrane or in the cytoplasm of root cells would have resulted in a signal cascade and transcription of defence related genes. This would have caused the early increases in callose deposition in all rootstocks at 6hpi, and the early production of phenolics in R0.38. Callose deposition may have hindered *Pc* from penetrating root cell walls and forming haustoria (Hardham and Blackman, 2018), assuming *Pc* was still in the biotrophic phase before 12 hpi (Backer *et al.*, 2015). However *Pc* was still able to grow through apoplastic spaces of the susceptible R0.38, because it reached the stele by 6 hpi, where gum deposits in the xylem were observed. Hence the susceptibility of R0.38 probably results from an inability to slow the apoplastic progression of *Pc*, which may indicate that it failed to successfully deploy apoplastic defences, which and R0.06 and R0.09 may have successfully done. At the proposed BNS of 12 hpi, R0.09 must have detected changes in *Pc* virulence and produced the sizable deposition of callose which possibly limited cell damage from *Pc*, and may be responsible for the documented resistance of R0.09. Although the anatomical study did show that *Pc* is capable of reaching stele in R0.09 at 12 hpi, *Pc* colonisation was comparatively much less than

seen in R0.38 at 6 hpi. The lack of significant ROS production did concur with the lack of lignin deposition as ROS are required for the oxidative polymerisation of lignin monolignols (Barros *et al.*, 2015), but the changes in antioxidants suggest there may have been some small changes in ROS which were possibly linked to defence signalling. The possible dephenolisation of lignin at 24 hpi adds support to the idea that *Pc* would be in necrotrophic phase by 24 hpi if it was actively degrading lignin.

This study has identified differences in the early physiological response of resistant and susceptible rootstocks to infection with *Pc*, and this is the first time quantitative callose and lignin assays have been performed on R0.09, R0.06 and R0.38 in response to *Pc* infection, and the first time that R0.38 has been examined with respect to *Pc* resistance mechanisms.

Future studies should focus on verifying the timing of BNS in *Pc* infected avocado to understand when *Pc* becomes more damaging, and which rootstocks respond the most defensively to BNS. The fact that resistant R0.06 deposited callose with a similar timing to the susceptible R0.38 rather than the resistant R0.09 means that the role of callose in resistance differs between the two resistant rootstocks. Therefore R0.09 and R0.06 should be used together in order to study the deposition of callose in response to *Pc* effectors or deposition after *Pc* infection and inhibition or gene knock out of avocado callose synthase. Callose deposition can be inhibited by 2-deoxy-D-glucose (Perumalla and Health, 1989), but as *Pc* produces its own β-glucan (Aronson *et al.*, 1967), careful optimisation needs to be performed to ensure that *Pc* growth is not directly affected by the inhibitor. As callose deposition in response to infection was already evident at 6 hpi in all rootstocks and *Pc* was observed in the stele of R0.38 at

this time, earlier time points than 6 hpi should be investigated to identify when the earliest deposition begins. The efficacy of apoplastic defences of R0.09, R0.06 and R0.38 should be investigated as it is probable that R0.38 is ineffective compared to the resistant rootstocks. An example of an apoplastic defence that could be investigated is the secretion of pathogenesis related proteins, which are a defence against pathogens (Delaunois et al., 2014), and these could be potentially explored using proteomic or gene expression analysis. If whole plant studies were to be used, the use of a soil free growing system could lower handling stress on plants when uprooting and replanting, but optimization would be required to ensure sufficient oxygen diffusion to prevent flooding stress of avocado roots (Schaffer *et al.*, 2013).

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Chapter 6

Appendix

A.1 Statistical Assumptions and Tests

The analysis of variance (ANOVA) and *t*-test assumptions for continuous data, independence of groups and independence of observations were satisfied by experimental technique (Dytham, 2011, Field 2013). For the remaining assumptions, namely the elimination of outliers, normality and equality of variance, the required statistical procedures were performed and are listed as follows. Firstly, for the elimination of outliers, data was plotted in box and whisker plots and whiskers were extended to one and a half times the interquartile range (Figure A1). All data points falling outside the range of whiskers were labelled as outliers and removed from each dataset. No more than a single outlier was found per dataset. Furthermore, to test the normality of the data, Q-Q plots and Shapiro-Wilk tests were used. The close clustering of data points to the straight line in the Q-Q plots was an indicator of normality in all datasets (Figure A2, 3, 4 and 5). The Shapiro-Wilk tests showed all p-values to be greater than 0.05, which indicated that data did not significantly differ from the normal distribution (Table A1). Lastly, to test for equality of variances, quotients were obtained by dividing the number of samples in the largest group by the number of samples in the smallest group, and as all quotients were less than 1.5, the data showed equality of variance (Table A2), (Pituch *et al.*, 2013). After analysis by ANOVA, pairwise comparisons using independent *t*-tests were performed, and listed with the differences in means (Table A3, A4, A5 and A6).

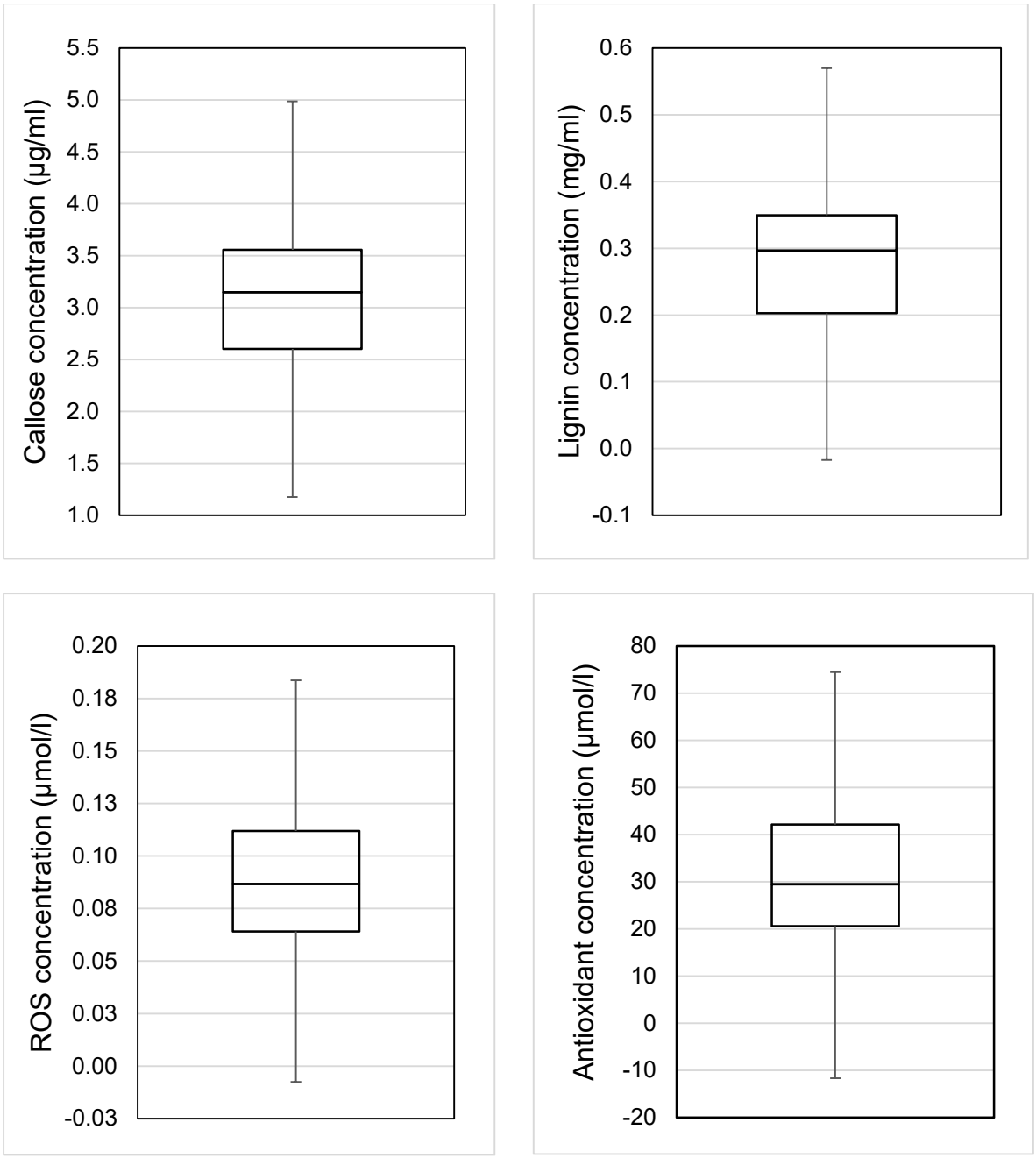

Figure A.1: Box and whisker plots of datasets. Top-left: callose, top-right: lignin, bottom-left: reactive oxygen species (ROS) and bottom right: antioxidants. Lines in boxes denote quartiles: the top line: Q3, middle line: Q2 (median) and bottom line: Q1.

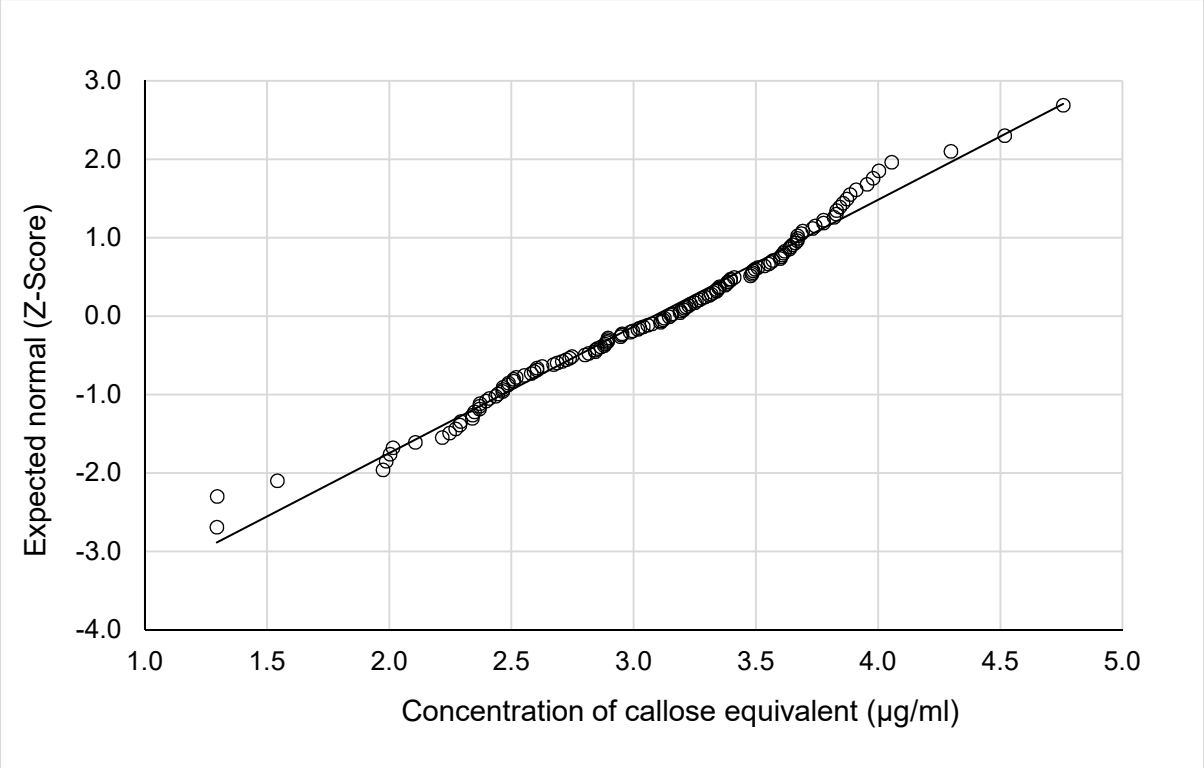

Figure A2: Q-Q plot of callose data showing proximity of data points to the line.

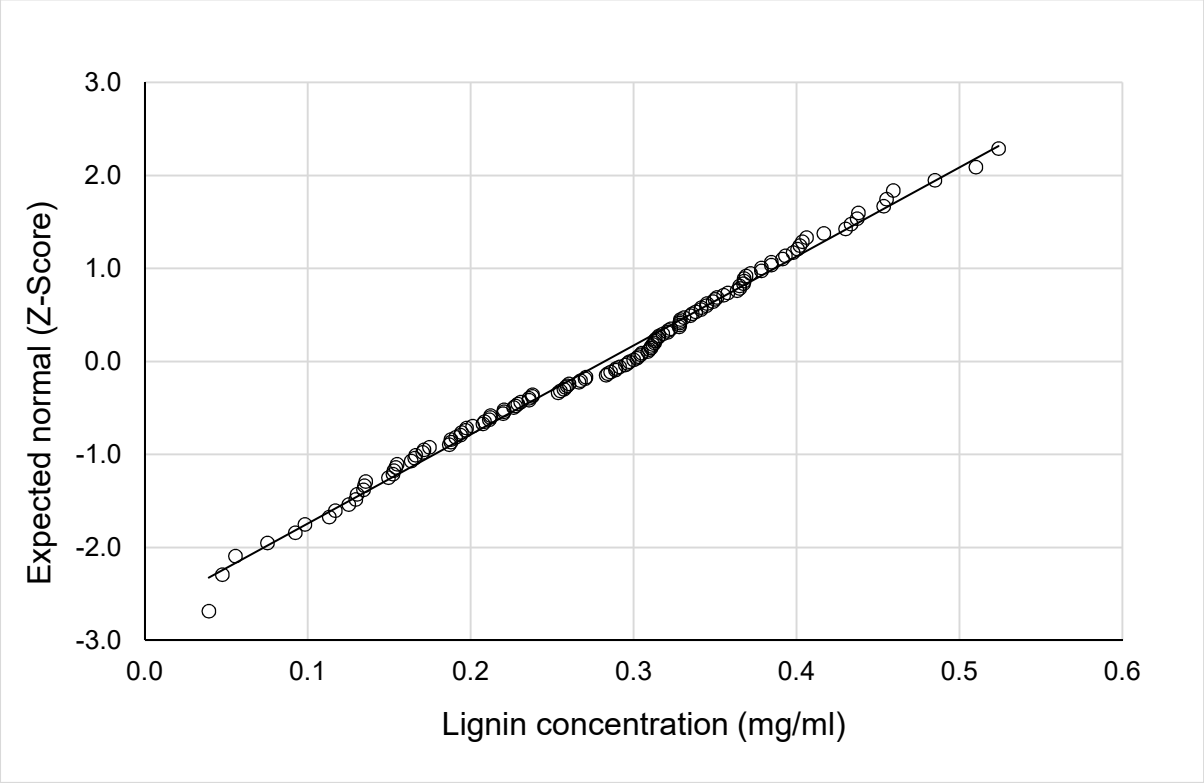

Figure A3: Q-Q plot of lignin data showing proximity of data points to the line.

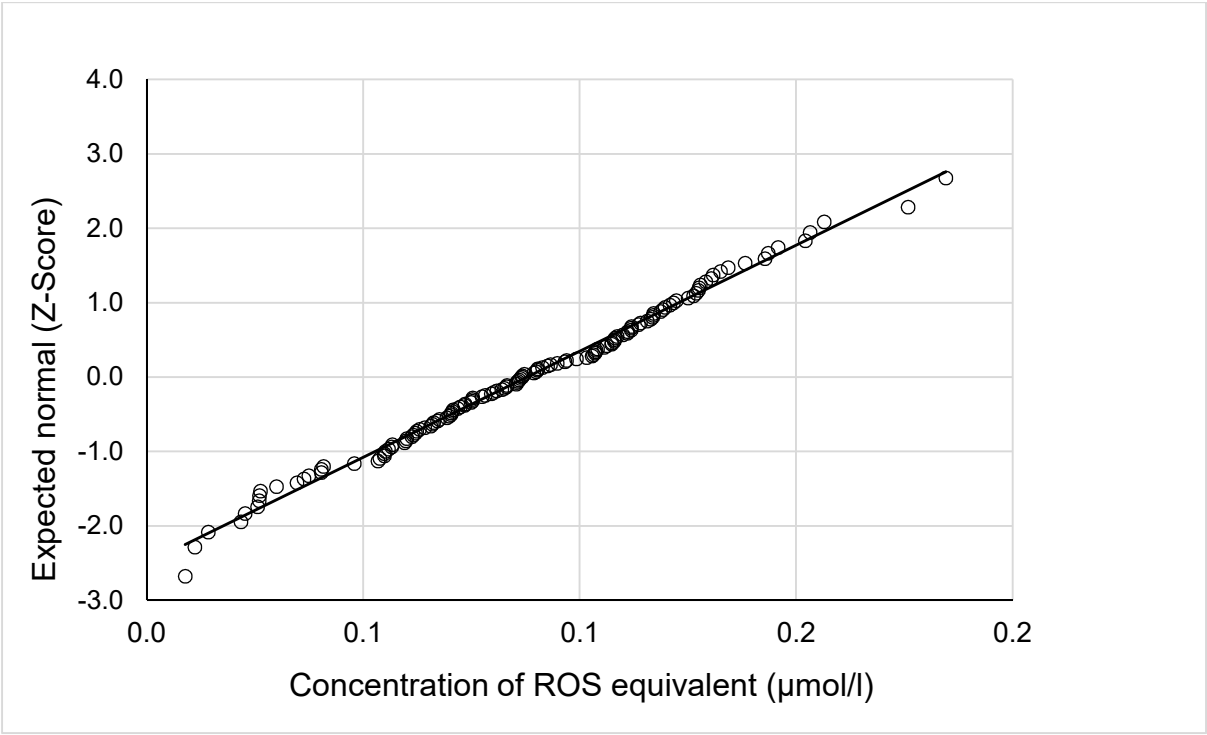

Figure A4: Q-Q plot of reactive oxygen species (ROS) data showing proximity of data points to the line.

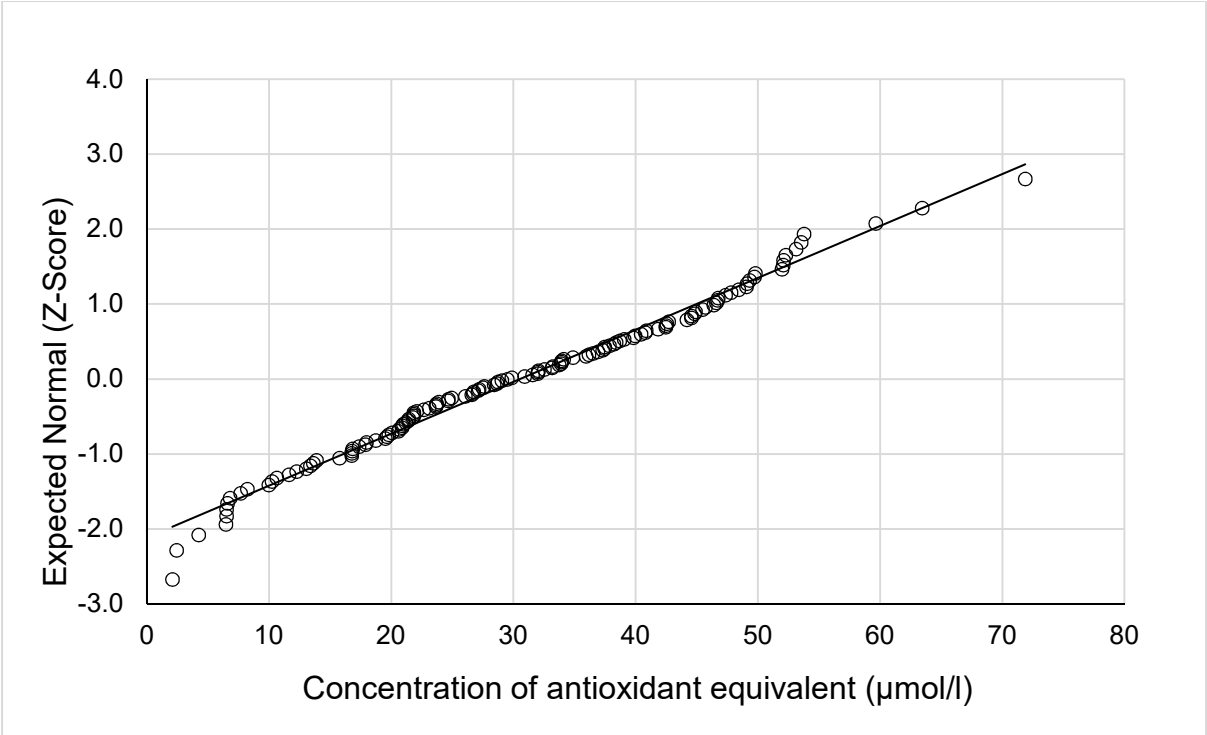

Figure A5: Q-Q plot of antioxidant data showing proximity of data points to the line.

Table A1: Shapiro-Wilk tests for normality.

	Degrees of freedom	**Sig.**
Callose data	140	0.159
Lignin data	136	0.412
ROS data	135	0.660
Antioxidant data	133	0.172

The p-values (Sig.) are greater than 0.05, showing normality of data.

Table A2: Factor group quotients.

	Quotient
Callose	1.00
Lignin	1.09
ROS	1.05
Antioxidants	1.08

All quotients are less than 1.5, showing the equality of variance.

Table A3: Callose differences between infected and uninfected sample means from 6 to 24 hours post infection.

	6 hpi	**9 hpi**	**12 hpi**	**24 hpi**
Infected R0.09	3.40±0.09[a]	3.06±0.06	3.33±0.11[a]	3.95±0.31[a]
Uninfected R0.09	2.94±0.18[c]	2.85±0.13	1.80±0.20[d]	2.45±0.04[d]
Difference in means	0.46	0.21	1.53	1.51
Infected R0.38	3.76±0.04[a]	3.38±0.07[a]	2.83±0.15	3.76±0.09[a]
Uninfected R0.38	3.05±0.19[c]	2.77±0.07[d]	2.80±0.24	2.39±0.10[d]
Difference in means	0.71	0.61	0.03	1.37
Infected R0.06	3.87±0.07[a]	3.48±0.05[a]	3.06±0.15	3.70±0.08[a]
Uninfected R0.06	3.18±0.24[c]	2.58±0.14[d]	3.15±0.10	2.47±0.09[d]
Difference in means	0.69	0.90	-0.09	1.23

The numbers listed in the first two rows for each rootstock are means followed by their standard error. The difference in means is the difference between infected and uninfected means. Superscripts are comparisons from independent *t*-tests at $\alpha = 0.05$, used to test the significance of the differences in means. Within single columns (but not between different rootstocks), an [a], [c] difference shows significance at the 5 % level, an [a], [d] difference shows high significance at the 1 % level and no difference in letters shows no significant difference.

Table A4: Lignin differences between infected and uninfected sample means from 6 to 24 hours post infection.

	6 hpi	**9 hpi**	**12 hpi**	**24 hpi**
Infected R0.09	0.28±0.02	0.22±0.04	0.35±0.06	0.16±0.05[a]
Uninfected R0.09	0.25±0.03	0.24±0.03	0.28±0.04	0.38±0.03[d]
Difference in means	0.033	-0.014	0.075	-0.211
Infected R0.38	0.26±0.03	0.20±0.03	0.37±0.05	0.18±0.07
Uninfected R0.38	0.27±0.04	0.22±0.02	0.33±0.02	0.32±0.05
Difference in means	-0.004	-0.016	0.040	-0.144
Infected R0.06	0.34±0.03	0.27±0.05	0.32±0.05	0.23±0.05[a]
Uninfected R0.06	0.27±0.04	0.32±0.02	0.32±0.03	0.35±0.01[b]
Difference in means	0.074	-0.054	-0.001	-0.122

The numbers listed in the first two rows for each rootstock are means followed by their standard error. The difference in means is the difference between infected and uninfected means. Superscripts are comparisons from independent *t*-tests at $\alpha = 0.05$, used to test the significance of the difference in means. Within single columns (but not between different rootstocks), an [a], [b] difference shows significance at the 10 % level, an [a], [d] difference shows high significance at the 1 % level and no difference in letters shows no significant difference.

Table A5: Reactive oxygen species differences between infected and uninfected sample means from 6 to 24 hours post infection.

	6 hpi	**9 hpi**	**12 hpi**	**24 hpi**
Infected R0.09	0.11±0.00	0.10±0.02	0.12±0.01	0.10±0.02
Uninfected R0.09	0.09±0.01	0.08±0.01	0.10±0.01	0.12±0.01
Difference in means	0.018	0.019	0.016	-0.016
Infected R0.38	0.06±0.02	0.05±0.02	0.08±0.02	0.06±0.01
Uninfected R0.38	0.07±0.01	0.07±0.01	0.08±0.00	0.07±0.02
Difference in means	-0.011	-0.018	-0.003	-0.013
Infected R0.06	0.10±0.01	0.11±0.02	0.10±0.02	0.08±0.02
Uninfected R0.06	0.08±0.01	0.08±0.01	0.09±0.02	0.11±0.01
Difference in means	0.023	0.028	0.004	-0.023

The numbers listed in the first two rows for each rootstock are means followed by their standard error. The difference in means is the difference between infected and uninfected means. Superscripts are comparisons from independent *t*-tests at $\alpha = 0.05$, used to test the significance of the difference in means. Within single columns (but not between different rootstocks), no difference in letters shows no significant difference.

Table A6: Total antioxidant differences between infected and uninfected sample means from 6 to 24 hours post infection.

	6 hpi	**9 hpi**	**12 hpi**	**24 hpi**
Infected R0.09	43.69±4.94	38.52±6.75	43.64±3.19[a]	31.29±4.19
Uninfected R0.09	50.04±5.05	30.50±2.24	34.72±3.23[b]	36.27±5.70
Difference in means	-6.34	8.02	8.92	-4.98
Infected R0.38	20.07±8.70[a]	26.89±4.72	32.86±4.26	22.97±8.93
Uninfected R0.38	41.03±6.52[b]	27.67±8.17	29.04±3.60	32.37±5.11
Difference in means	-20.95	-0.78	3.82	-9.39
Infected R0.06	17.28±4.73	19.17±4.62[a]	26.13±5.84	15.90±2.73
Uninfected R0.06	23.91±5.64	39.61±5.89[c]	21.34±1.20	20.49±4.23
Difference in means	-6.63	-20.44	4.79	-4.59

The numbers listed in the first two rows for each rootstock are means followed by their standard error. The difference in means is the difference between infected and uninfected means. Superscripts are comparisons from independent *t*-tests at $\alpha = 0.05$, used to test the significance of the differences in means. Within single columns (but not between different rootstocks), an [a], [b] difference shows significance at the 10 % level, an [a], [c] difference shows significance at the 5 % level and no difference in letters shows no significant difference.

A.2 References

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