Physiological responses of South African avocado rootstocks to *Rosellinia necatrix* and potential control strategies

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Declaration

I, Phinda Magagula, hereby declare that this thesis, submitted to the University of Pretoria for the degree MSc Plant Science, contains my work and that the content within the thesis has not been previously submitted to any other University.

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Summary

Rosellinia necatrix Berl. Ex Prill. is a fungal plant pathogen and the causal agent of White Root Rot (WRR), which has caused economic losses in forests and agricultural industries in temperate and subtropical areas. The pathogen was detected and identified in 2016 in South African avocado orchards, causing decline and death of avocado plants. At present, the detection of *R. necatrix* relies on human visual symptom detection, morphological identification, and real-time PCR confirmation assays. PCR is a molecular tool used for detection of *R. necatrix*. Still, its reliability is highly dependent on the selection of infected samples for assay, which can be scarce and challenging in newly infected plants. Therefore, early detection of WRR before stress symptoms are evident and rapid response are necessary to limit the spread of the pathogen. Lack of effective post-infection control strategies necessitates early detection of WRR to combat the further spread of the disease.

The study aimed to investigate physiological parameters, including leaf gas exchange and chlorophyll fluorescence, to determine the onset of stress in avocado plants following infection with *R. necatrix*. Also, to examine the effect of biological agents and chemical products in avocado plants infected with *R. necatrix*. WRR symptoms were assessed using human visual detection and a disease scoring system. The plants were monitored for signs of stress using the open path LI6400XT photosynthesis machine. PCR confirmation assays were conducted at the end of the trials to confirm the presence of *R. necatrix*.

WRR symptoms were evident at 35 days post-infection (dpi) in infected plants of all the rootstocks and confirmed death at 60 days, while control plants showed no signs of stress throughout the trial. The effect of WRR caused necrosis and root rot, which was related to plants' failure to absorb water, therefore, they closed their stomata. Stomatal closure caused a reduction in CO_2 availability of the leaf, which affected net CO_2 assimilation (A_N) and limited carbon fixation. This resulted in a reduction of dry mass production and allocation in infected plants which were significantly different to control plants.

The significant decrease in A_N and stomatal conductance (g_s) of infected plants was matched by chlorophyll flouerescence parameters and these were observed at 35 dpi once stress symptoms were evident as wilting of the leaves. Stomatal limitations were the main factors responsible for reducing A_N in infected plants relative to the control plants. The evidence of lower values of intercellular CO_2 to atmospheric CO_2 concentration ratio (C_i/C_a) at post-symptomatic stage supported that a decline in A_N was due to stomatal limitation. A significant decrease in the maximum quantum efficiency of photosystem II (F_v/F_m ratio) at 28 dpi was observed between infected and control plants. This early reduction in F_v/F_m ratio may be due to photo-inhibition as a result of low intercellular CO_2 in the leaves of infected plants under saturating light relative to control plants. Assessing photosynthetic parameters may be useful in the detection of the onset of stress in plants for further investigation under greenhouse conditions. This strategy of stress monitoring in plants provides a baseline study for field work to minimize response time and exposure of the pathogen.

WRR control was investigated using biological agents, fluazinam, and chloropicrin in Chapter 3. Two biological agents (*Trichoderma* and B-Rus), fluazinam and chloropicrin were able to inhibit *R. necatrix* when grown *in vitro*. *Trichoderma* and B-Rus further suppressed WRR disease symptoms in plants when applied before infection of the plants with *R. necatrix* in a greenhouse trial. Infected plants showed a decrease in A_N, and g_s relative to all the treatments because of stomatal limitation. Plants treated with B-Rus and *Trichoderma* were able to sustain dry mass production and A_N compared to plants treated with Beta-Bak, Mity-Gro^{TM} and *R. necatrix*-infected control plants. Fluazinam was applied after infecting the plants with *R. necatrix* under greenhouse conditions and showed a reduction in WRR disease symptoms. Chloropicrin showed the potential of killing *R. necatrix* when used as a pre-plant treatment. No viable *R. necatrix* was isolated from chloropicrin treated soil and plants appeared healthy, and no traces of white mycelia were found on the roots. This study is a critical step in pursuit of field level work of an integrated management approach for WRR disease in avocado orchards.

Preface

Early detection of the introduction of emerging threats followed by a rapid response to expose the pathogens is the most important strategy to minimize damage to agricultural production. For plant diseases, the lack of efficient post-infection treatments and the inability to prevent infection, dictate that early detection and response are critical. The increased occurrences of emergent diseases like White Root Rot (WRR) are posing a challenge to avocado production in countries where the disease is presence. This disease is increasingly becoming a global threat after Phytophthora Root Rot (PRR) in avocado production areas. WRR disease is a problem to many woody plants which affects their water relations and limits photosynthesis, therefore reduction in dry mass production. Currently, no commercial tolerant or resistant rootstock against WRR is available and control strategies against the disease have been inefficient to completely eradicate the pathogen. Discovery of early stress detectors in plants will prompt further investigation and implementation of control measures before visual WRR symptoms are evident. The availability of biological agents and chemical control products will provide an additional tool to inhibit and treat WRR in avocado plants. The conclusions of this thesis will aid in providing of an integrated management approach against WRR in avocado orchards in South Africa.

The research work presented in this thesis is based on the laboratory and greenhouse trials conducted by the Avocado Research Programme (ARP) at the University of Pretoria, South Africa. The thesis is presented in four independent chapters, and therefore some repetition was inevitable.

Chapter 1 provides a literature review on *R. necatrix* as the causal agent of WRR on woody plants, including avocado. This includes describing WRR symptoms and the infection strategy of the pathogen. This review also focuses on WRR control methods currently employed to eradicate WRR disease. It concludes with a section on plant physiological responses to *R. necatrix,* and the focus area was the use of photosynthesis processes as an early detection method of stress in plants infected with *R. necatrix*.

Chapter 2 is the first research chapter of this thesis. The parameters of leaf gas exchange and chlorophyll fluorescence were assessed to monitor stress in plants under greenhouse conditions of three avocado rootstocks infected with *R. necatrix* using a photosynthesis LI6400XT portable machine. The results from this chapter suggest that stomatal limitation was the main factor limiting net CO_2 assimilation which further limited biomass or dry mass production and allocation in infected plants relative to uninfected control plants of all the rootstocks. The results of this chapter provided reliable detection tools of *R. necatrix* under greenhouse conditions.

In **Chapter 3**, biological agents, fluazinam and chloropicrin were evaluated in the laboratory and *in in vivo* in the greenhouse against *R. necatrix.* Also, physiological analyses were conducted to understand the influence of biological control agents on net CO_2 assimilation and stomatal conductance during *R. necatrix* infection. The results provided information on control products available against *R. necatrix*. This information can be used to facilitate registration of control products against *R. necatrix* in avocado orchards.

Chapter 4 is the general conclusion of the thesis based on the results presented in this study. This chapter focused on suggesting an integrated management approach using different control methods and early detection techniques of stress in avocado orchards. The integrated management approach will provide a tool that will exploit all the advantages of the control products and detection techniques to combat WRR in avocado orchards and ensure the sustainability of avocado production in South Africa.

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List of abbreviations

Anova	Analysis of variance
C_a	Atmospheric CO_2 concentration
ARP	Avocado Research Programme
ARD	Apple replant disease
bp	Base pair
BB	Beta-Bak
BCAs	Biological control agents
BLAST	Basic Local Alignment Search Tool
BR	B-Rus
CEF	Candidate effector protein
CO_2	Carbon dioxide
cm	Centimeter
CTAB	Cetyl trimethylammonium bromide
$A\text{-}C_i$	CO_2 assimilation to increasing intercellular CO_2 concentration
A_N	CO_2 assimilation
CMV	Cucumber mosaic virus
Rd	Daytime respiration
Dpi	Days post-inoculation/ Days post-infection
°C	Degree Celsius
DNA	Deoxyribonucleic acid
EBS01	Electrode boiler solution

Φ_{PSS}	Efficiency of photosystem II
et al.	*Et alia* meaning ‘and others’
FABI	Forestry and Agricultural Biotechnology Institute
FLU	Fluazinam
g/L	Grams per liter
h	hours
F_o	Initial minimum fluorescence
IPM	Integrated pest management
C_i	Intercellular CO_2
C_i/C_a ratio	Intercellular CO_2 to atmospheric CO_2 concentration
iWUE	Intrinsic water use efficiency
LSD	Least Significant Difference
F_m	Maximum fluorescence when PSII centers are closed
$F_{m'}$	Maximum fluorescence yield of pre-illuminated sample
F_v/F_m ratio	Maximum quantum efficiency of photosystem II
$V_{c\ max}$	Maximum rate of RuBP carboxylation
J_{max}	Maximum rate of electron transport driving RuBP regeneration
µL	Microliter
µL/mL	Microliter per milliliter
µmol CO_2 mol^{-1}	Micromole CO_2 per mole
µmol $m^{-2}s^{-1}$	Micromole per square meter and per second
µmol quanta $m^{-2}s^{-1}$	Micromole quanta per square meter and per second
$MJm^{-2}day^{-1}$	Megajoule per square metre per day
m	Metres

mL	Milliliter
mL/L	milliliter per liter
mL/min	Milliliter per minute
mm	Millimeter
mg	Milligrams
mg/kg	Milligrams per kilogram
min	Minutes
Mg	Megagram
MG	Mity-Gro^{TM}
NPQ	Non-photochemical quenching
O_2	Oxygen
%	Percentage
PI	Percentage inhibition
PSII	Photosystem II
PAR	Photosynthetic Active Radiation
PPFD	Photosynthetic Photon Flux Density
qP	Photochemical quenching
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PRR	Phytophthora Root Rot
RCBD	Randomized complete block design
R:S	Root:shoot ratio
Rn	*Rosellinia necatrix*
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase

RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RuBP	Rubulose-1,5-bisphosphate
RWC	Relative water content
SEM	Scanning electron microscopy
SAS	Statistical analysis
±SE	Standard Error
F_s	Steady-state chlorophyll fluorescence
F_s/F_o	steady-state yield fluorescence (F_s) normalized to initial minimal fluorescence (F_o)
g_s	Stomatal conductance
Tricho	*Trichoderma*
V	Volts
H_2O	Water
WRR	White Root Rot

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CHAPTER 1

Rosellinia necatrix, an emergent threat of White Root Rot on avocado

Introduction

The projected human population by 2050 is 10 billion and crop production in the next 50 years should double to cope with the increased food demand (Hunter et al. 2017). The world's fast-growing human population presents a significant strain on food security, and currently, plant pathogens and pests threaten these food crops (Ghorbanpour et al. 2018). Avocado (*Persea americana* Mill*.*) is among the food crops affected by pest and diseases worldwide. The demand for the fruit crop has increased over the decades because of health benefits to humans. The most important pathogen of avocado worldwide is still *Phytophthora cinnamomi* Rands (Zentmyer 1980, Reeksting et al. 2014), whilst *Rosellinia necatrix* Berl. Ex. Prill. is also becoming more important. *R. necatrix* is the causal agent of White Root Rot (WRR) and is most prevalent in Spain and Israel (Pliego et al. 2012), and now also reported in major avocado productions areas in South Africa (van den Berg et al. 2018).

The soil-borne ascomycete pathogen attacks the root system of plants which results in necrosis of feeder roots as the root tissues decay showing brittleness and turning black (Pliego et al. 2009, Pliego et al. 2012). This phenomenon restricts water and nutrient uptake by plants, leading to leaf wilting and eventual tree death (Martínez-Ferri et al. 2016). The pathogen invades both epidermal and cortical cells, collapsing the vascular system of the plant (Pliego et al. 2009). *R. necatrix* produces a toxin (Cytochalasin E) during infection which has been associated with virulence and pathogenicity in host-pathogen interactions (Kimura et al. 1989, Edwards et al. 2001, Kshirsagar et al. 2001, Martínez-Ferri et al. 2016). The transcriptome analysis of *R. necatrix* during infection of susceptible 'Dusa®' plants has identified genes involved in the production of toxins, detoxification and transport of toxic substances, hormone biosynthesis, plant cell wall degradation and gene silencing (Zumaquero et al. 2019a). In the same study, candidate effector proteins (CEP) were predicted during *R. necatrix* infection of avocado roots. Another study identified genes involved in osmotic and salt stress in BG83 rootstock tolerant to WRR which could show that *R. necatrix* penetration into the roots of avocado is related to osmotic effects (Zumaquero et al. 2019b).

The present detection methods of *R. necatrix* relies on microscopic and molecular methods (Pérez-Bueno et al. 2016). These methods have limitations at field level because they are time-consuming, costly, need human resources for sampling and delay implementation of possible control measures. In large orchards, some infected trees can go undetected until visible above-ground WRR symptoms are evident. Real-time PCR is an early detection tool for pre-symptomatic infection. As consequences of time taken to detect WRR, infected plants can serve as an inoculum source for disease spread to surrounding plants. There is a need for reliable, sensitive, easy-to-use detection methods for fungal pathogens on a large scale in commercial orchards (Ray et al. 2017).

R. necatrix remains a challenge to control because it is resistant to most fungicides, survives in acidic soils and dead wood material and no commercial resistant or tolerant avocado rootstocks are available (Pliego et al. 2012). Current control strategies include physical control which makes use of soil solarization and rogueing of infected plant materials (López-Herrera et al. 1999), and chemical control using broad-spectrum fungicides such as fluazinam (López-Herrera and Zea-Bonilla 2007, Arjona-López et al. 2020). Biological control using *Trichoderma* and rhizobacteria species have demonstrated inhibition against *R. necatrix* (Ruano-Rosa et al. 2014b). These control strategies have shown to be ineffective in completely eradicating *R. necatrix* from infected areas*.* The lack of effective post-infection control dictates that early detection of stress in plants should be considered for rapid response against WRR disease in avocado orchards.

This review will discuss *R. necatrix* as an important pathogen for woody plants and how it causes WRR in avocado. Also, the review will look at the control strategies currently available and their reported efficacy against the disease. Lastly, it will focus on the physiological responses of avocado plants to *R. necatrix* and how this can be exploited for early WRR detection.

R. necatrix **Berl. Ex. Prill: the causal agent of White Root Rot (WRR)**

Taxonomy

Taxonomy is the science of naming, defining, and classifying groups of biological organisms on the bases of characteristics. The ascomycete WRR pathogen (class: Pyrenomycete/Sordanomycete) was classified by de Notoris in 1844 as Eurotiomycetes due to the aspects of its fruiting body which unfortunately also contributed to scientist's failure to recognise it. Later it was reclassified as Pyrenomycetes. The genus *Rosellinia* belongs to an important family known as Xylariaceae with more than 100 species that are root pathogens (Petrini 1993). The pathogen was previously known as *Rhizomorpha necatrix* when found, causing WRR on grapes (*Vitis vinifera* L.) in Germany and France (Hartig 1883). The study analysed conidial morphology and suggested the teleomorph of the pathogen could belong to *Rosellinia*. A descriptive analysis of morphology was further conducted on ascocarps of *Rosellinia aquila* and included *Dematophora necatrix* in the genus *Rosellinia* (Berlese 1892). The classification of the pathogen was also evaluated by obtaining perithecia from fruit tree roots infected with *D. necatrix* and named the teleomorph, *R. necatrix* (Prillieux 1902). Interest in this pathogen developed over the years which led to the first descriptive analysis of the teleomorph (Prilleux 1904). Several years later, the classification of the fungal taxonomy still does not have the different names of the teleomorph and anamorph (Nakamura et al. 2000).

Host and geographic distribution

A host is a plant that harbours a parasitic or a mutualistic pathogen. *R. necatrix* has been found to occur in many hosts over the years as a parasite and has become a global threat to many forest trees, horticultural and ornamental plants (Pliego et al. 2012). The first report of the list of host plants was released in 1959 with 170 species, including 63 genera which belong to 30 families and algae species (Khan 1959, Pliego et al. 2012). The economic importance of this pathogen has prompted researchers to continue searching for new host species, and the list grew to 197 different species (Arakawa et al. 2002, Pliego et al. 2012). Currently, the fungus host list is at 475 species (Farr and Rossman 2020). Some high-value crops affected by this pathogen included avocado (*P. americana*), mango (*Mangifera indica*), citrus (*Citrus spp.*), apple (*Malus domestica*), pear (*Pyrus communis*) and coffee (*Coffea spp.*) (Pliego et al. 2012).

The geographic distribution of the pathogen refers to the outcomes of the dynamic processes involving host availability, evolutionary change, the suitability of climatic conditions and susceptibility (Shaw and Osborne 2011). The origin of *R. necatrix* is unknown, and the route by which it has spread is not well understood but believed to be via infected plant materials (Mantell and Wheeler 1973, Pliego et al. 2012). In the 1950s, the pathogen was reported to be a problem in areas including Africa and Japan, limiting many crop species (Abe and Kono 1956). The presence of *R. necatrix* in apple orchards was reported four decades ago in South Africa (Van Der Merwe and Matthee 1974), before the first report in avocado orchards (van den Berg et al. 2018). The fungus was later reported in Mozambique affecting several important trees (United Kingdom CAB International 1987). The pathogen was further reported infecting poplar and many fruit trees in Italy (Cellerino et al. 1988). The following year, *R. necatrix* was first published in Southern Spain infecting avocado plants in 1989 (López Herrera 1989). A few years later, France and Portugal reported the pathogen causing a problem in apple trees (De Sousa et al. 1995) and later in the year, Brazil also reported the pathogen (Denardi and Bretón 1995). Recently, the pathogen was reported infecting avocado plants in South Africa (van den Berg et al. 2018). The different reports worldwide show that WRR is an important pathogen in avocado and other fruit crops (Pliego et al. 2012). However, limited information is known about the spread of the pathogen on avocado specifically in South Africa.

Sexual and asexual reproduction

The most diverse eukaryotic kingdom at present is the fungi (Hawksworth and Lücking 2017). The diversity has led to different life cycles with a high level of reproductive plasticity (Wilson et al. 2019). Fungal species have a wide variety of mechanisms of both sexual and asexual reproduction. *R. necatrix* poses both sexual and asexual reproductive life cycles (Figure 1.1), which promotes its development in different climatic conditions and a wide host range (Pliego et al. 2012).

Sexual reproduction in ascomycetes is introduced upon the recognition of different factors that change the vegetative mycelia into sexual-competent tissue (Wilson et al. 2019). The sexual reproductive structures of *R. necatrix* are ascospores (Figure 1.1) (Pliego et al. 2012). In fungi, sexual reproduction occurs in response to unfavourable conditions like drought and acidic soil. Sexual reproduction in fungi uses mating types known as master regulators of sex (Dyer et al. 2016). There are two mating types involved. When both mating types are present in the same mycelium, it is called homothallic, or self-fertile. *Rosellinia*-mating-type genes have not yet been identified (Kanda et al. 2003). The life cycle of this pathogen might be homothallic (Aimi et al. 2002). However, *R. necatrix* showed a heterothallic life cycle when assessed using a DNA polymorphism analysis of ascospore progenies (Ikeda et al. 2011). Heterothallic mycelia require two different, but compatible, mycelia to reproduce sexually. Infected plant roots thus can develop synnemata which later produce conidia as spermatia, that further spreads and produces perithecia (Nakamura et al. 2002, Ikeda et al. 2011).

The most common mode of asexual reproduction in fungi is through the formation of spores (Kanda et al. 2003), which are produced by one parent only (through mitosis) and are genetically identical to that parent. Spores allow fungi to distribute and colonize new environments. *R. necatrix* has two asexual reproductive structures, namely, chlamydospores and conidiospores (Figure 1.1) (Pérez-Jiménez 2006, Pliego et al. 2012). Chlamydospores can rarely be found under natural conditions where the pathogen reproduces asexually (Pérez-Jiménez et al. 2003). Chlamydospores are different from conidia because they are formed from pyriform swellings under which the protoplast is condensed and encased in a cell wall (Makambila 1976, De Labrouhe 1986, Pliego et al. 2012), while conidia initiate at the end of the synnemata conidiogenous cells (Pérez-Jiménez 2006). The synnemata structure can be observed in tree roots or soils previously infected with *R. necatrix,* and their development can be largely dependent on environmental growth conditions (Pérez-Jiménez 2006).

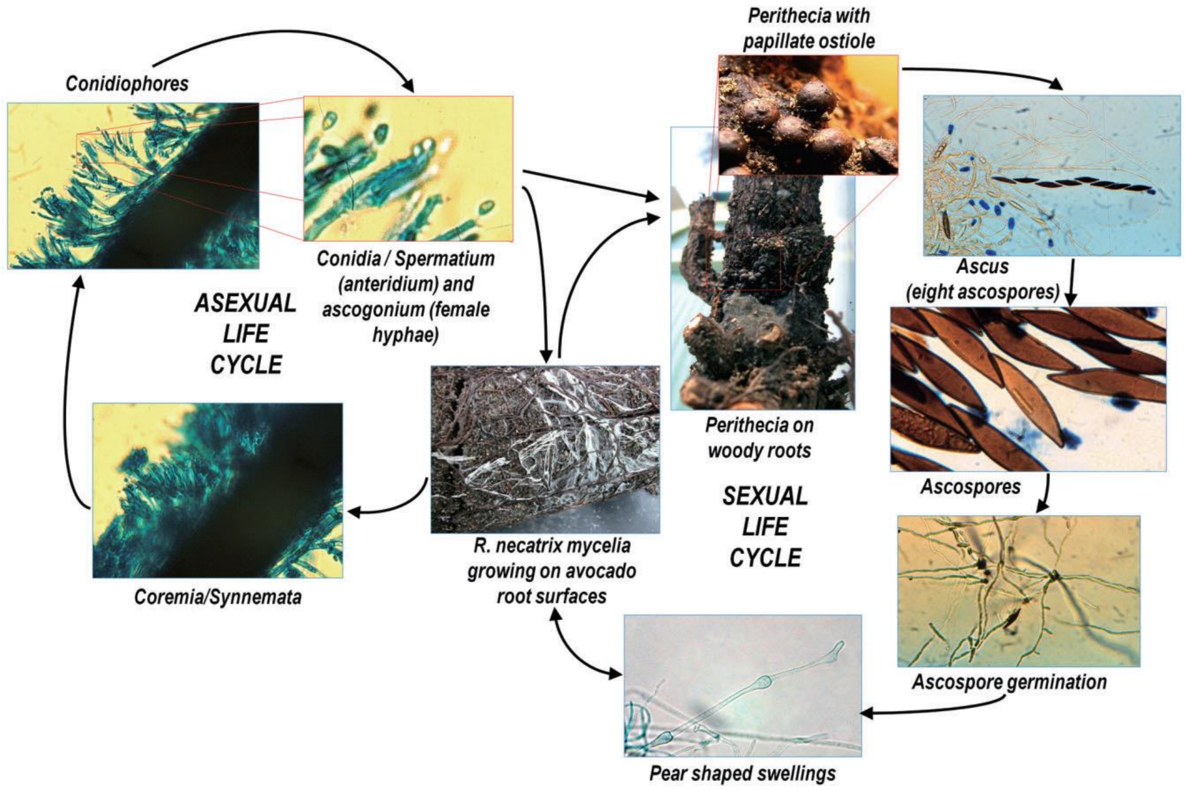

Figure 1.1: The Life cycle of *Rosellinia necatrix*, covering the sexual and asexual aspects of the cycle. The asexual life cycle occurs via two different spore types, chlamydospores and conidiospores. The sexual life cycle occurs only via ascospores which are found in infected tissues of plants (Pliego et al. 2012).

R. necatrix *infection strategy*

The WRR pathogen survives as a necrotroph. It derives energy from dead cells; they invade and kill plant tissue rapidly and then live saprophytically on the dead remains (Pliego et al. 2012). The pathogen can switch between saprophytic and pathogenic lifestyles which allows its survival for an extended period. The pathogen switches to a saprophytic life cycle in the absence of the host and unfavourable conditions, while reverting to a pathogenic life cycle when a suitable host is present (Pliego et al. 2012).

The first report on WRR development was described in mulberry trees (*Morus rubra*) (Sakurai 1952, Pérez-Jiménez 2006). The study showed that the fungus invades young roots that still lack secondary tissues by dissolving the cell wall. In older roots, the fungus invades inner tissues through natural openings as hyphal strands. As the fungus progresses in the natural openings of the root system, it causes wounds by breaking them and affected cells demonstrate brownish colouration and necrosis (Pérez-Jiménez 2006). Following the development of the wound periderm in the cortical tissues, the hyphae strands further break the periderm and invade healthy tissues. When the hyphae reach the wood tissue, they invade the vessels and cells through medullary rays (Pérez-Jiménez 2006). Other studies showed a fan-like mycelial aggregation of *R. necatrix* invading the cork layers of different plant species including jonquil (*Narcissus jonquilla* L.), Japanese pittosporum (*Pittosporum tobira*) and creeping wood sorrel (*Oxalis corniculata*) (Mantell and Wheeler 1973, Pérez-Jiménez 2006). These layers were damaged, and mycelia were found in the xylem vessels blocking the uptake of water and nutrients.

The penetration of the pathogen was described in apple roots through artificial infection (De Labrouhe 1982) and later reviewed by Pérez-Jiménez (2006). The penetration of *R. necatrix* in apple roots was analysed at different stages (Pérez-Jiménez 2006). Firstly, the pathogen progresses in the soil as a diffuse mycelium. Secondly, the fungus penetrates young tissues without secondary development through formation of mycelial aggregates. These structures are formed by hyphae which occur in the root surfaces. The fungus penetrates the parenchyma cortical without affecting the cell wall. In older tissues, penetration occurs by formation of a ‘sclerotium of penetration’ (Pérez-Jiménez 2006). These sclerotia are structures of small black grains with a black cortex of pseudoparechyma with isodiametric melanized cells and a white medulla of prosenchyma with hyphae converging to the middle of the sclerotium (Pérez-Jiménez 2006). This penetration also occurs without affecting the cell walls. In older tissues, white mycelia develop between the suber and phelloderm, which can be observed as white rays around the penetration point below sclerotium (Pérez-Jiménez 2006). In this subcortical strand the hyphae grow in all directions and form the typical fan mycelia of WRR (Pliego et al. 2012). This is then followed by destruction of the cell walls and disorganization of the root tissues. Lastly, the mycelia around the necrotic roots change to a dark colour and progress to infect healthy tissues (Pérez-Jiménez 2006). The death of plants is caused by the rotting of the whole root system and the roots completely fail to absorb water and nutrients from the soil (Pérez-Jiménez 2006, Pliego et al. 2012).

R. necatrix infection strategy on avocado roots has been observed using microscopic visualization of a virulent *R. necatrix* isolate CH53-GFP, transformed with a GFP fluorescent marker (Pliego et al. 2009). The penetration of the pathogen was found to take place in the crown region, including natural openings and junctions between epidermal cells. The study revealed that penetration of *R. necatrix* was not observed at the root tip along the secondary roots. This suggested that the penetration of *R. necatrix* followed a different pattern especially when compared with other fungi such as *Fusarium oxysporum* f. sp. *radices-lycopersici* (Lagopodi et al. 2002). Additionally, it is evident that *R. necatrix* produces toxins *in vitro* which could be related to pathogenicity and virulence (De Labrouhe 1982, Pliego et al. 2012). However, it is still unclear whether these toxins are involved in WRR symptom development and death of the plants. Using confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM), a clear view of *R. necatrix* invading the avocado root tissues was observed (Figure 1.2A, B and C) (Pliego et al. 2009). It was also shown that the pathogen penetrates both the primary and secondary xylem of avocado root tissues (Figure1.2D) (Pliego et al. 2009), which agrees with Mantell and Wheeler (1973) on jonquil roots infected by other *Rosellinia* strains. Following the invasion of the mycelia at the epidermal and cortical cells of the roots, cells collapsed, and hyphae continued to grow reaching the xylem and phloem vessels (Figure 1.3A). However, a different aggregation of fungal mycelia appeared invading the cortical cell layers (Figure 1.3B). The study suggested that SEM images supported by the results of CLSM images, for the first time, allowed a detailed visualization of the hyphal network of *R. necatrix* through epidermal, cortical, and vascular cells. This study also included the observation of the fusion between branches of the same hyphae and branching points of the pathogen (Figure 1.3C and D) (Pliego et al. 2009).

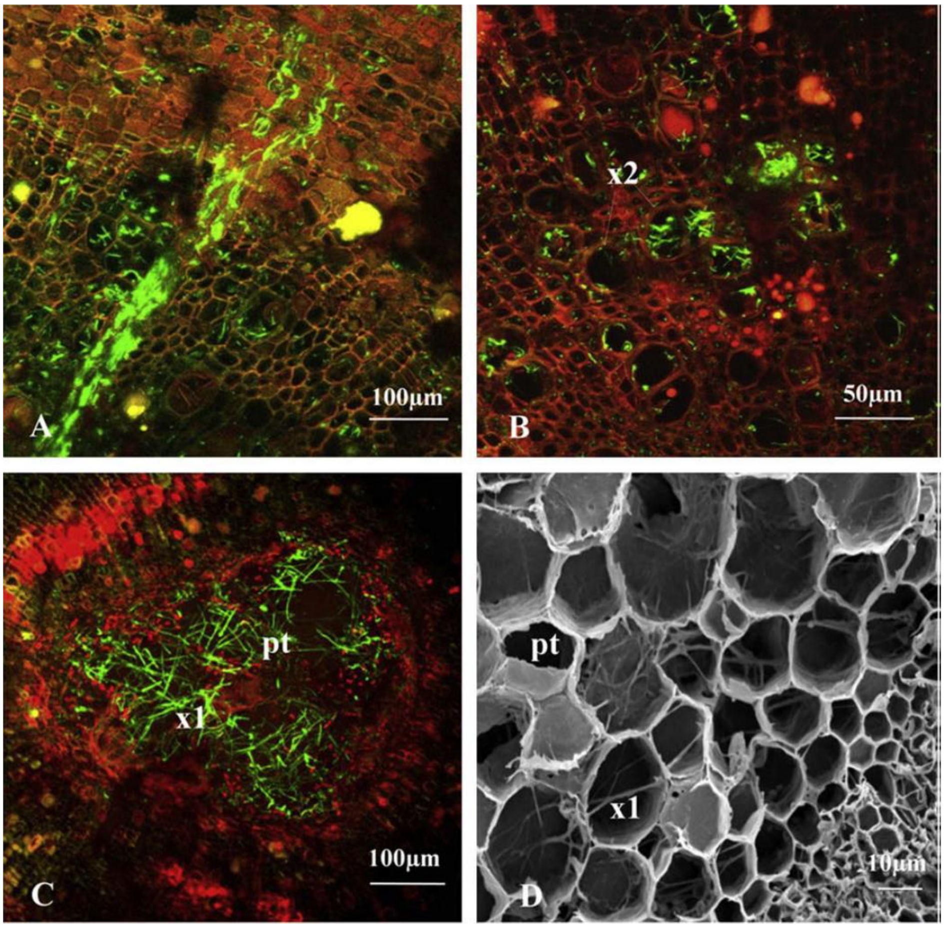

Figure 1.2: The visualization of advanced stages of *Rosellinia necatrix* CH53-GFP proliferation in 6-month-old avocado plant roots with secondary growth (A, B and C) Confocal laser scanning microscopy images of *R. necatrix*. (A) *R. necatrix* hyphal strand growing throughout secondary xylem vessels; (B) Detailed image of secondary xylem vessels invaded by fungal hyphae; (C) *R. necatrix* mycelia invading the pith and (D) Scanning electron microscopy image of *R. necatrix* hyphal network invading the primary xylem (Pliego et al. 2009).

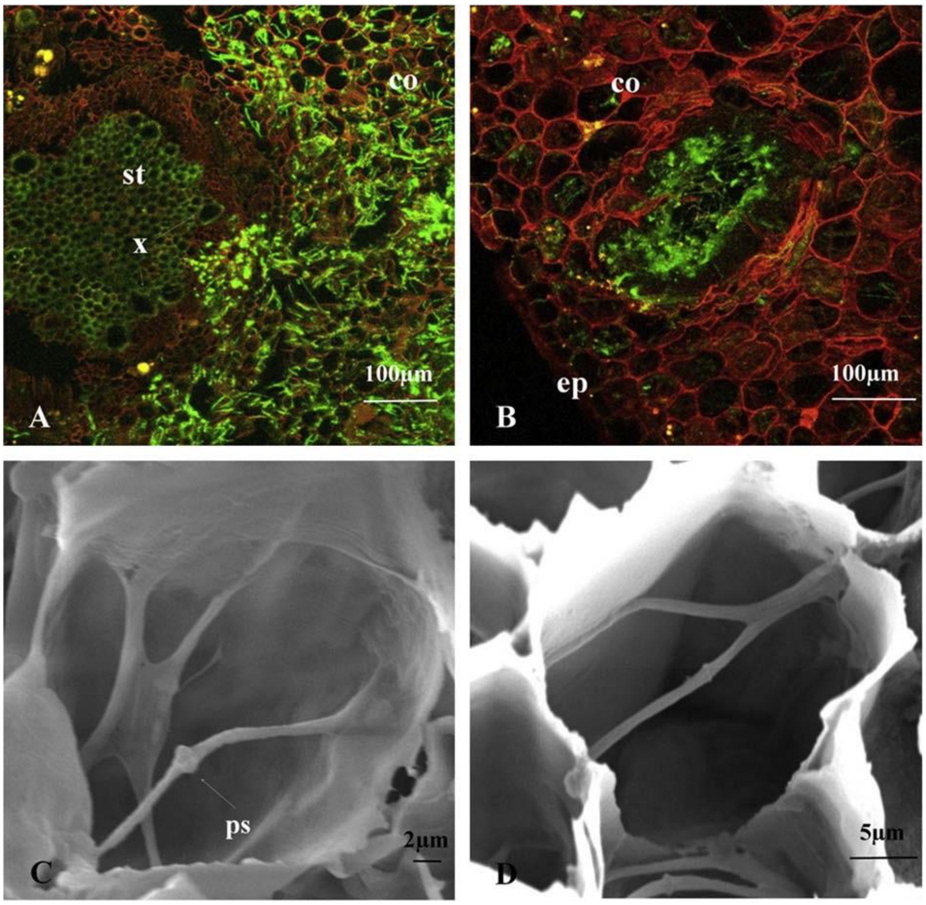

Figure 1.3: The visualization of advanced stages of *Rosellinia necatrix* CH53-GFP proliferation in 6-month-old avocado plant roots with primary growth (A and B) Confocal laser scanning microscopy images of *R. necatrix*. (A) *R. necatrix* hyphal invading cortical cells and reaching the stele; (B) Detail image of a mycelial aggregate in the cortical layer; (C) *R. necatrix* hyphae displaying anastomosis and pyriform swellings and (D) branching of fungal hyphae during intracellular growth (Pliego et al. 2009).

WRR disease symptoms

R. necatrix invades the roots of plants leading to a decline in vigour and rot of the under-ground parts (Pliego et al. 2012). The pathogen attacks healthy avocado roots without showing above-ground symptoms which makes it difficult to diagnose the pathogen early enough, before it causes severe damage to the plant (Pérez-Jiménez 2006, Pliego et al. 2012). The base of the trunk at the soil level can demonstrate signs of black wet rot, as well as rotting of the roots. The disease symptoms are highly severe under wet soils and high-temperature conditions (Van Der Merwe and Matthee 1974). Hyphal strands and cords are also visible on the soil surface around the root collar of the infected plant (Figure 1.4C, D and E) (Pliego et al. 2012).

The development of above-ground symptoms depends on the health of the plant, the inoculum load in the roots and the environmental conditions (López-Herrera and Melero-Vara 1992, Pérez-Jiménez 2006). As WRR progresses general symptoms include; leaf yellowing, small leaves, premature leaf fall, cessation of root growth and small, shrunken fruits and rapid death of plants where brown/black leaves are evident (Figure 1.4B) and fruit remain on the tree (Pliego et al. 2012). Healthy avocado plants will appear with green leaves and no sign of stress (Figure1.4A).

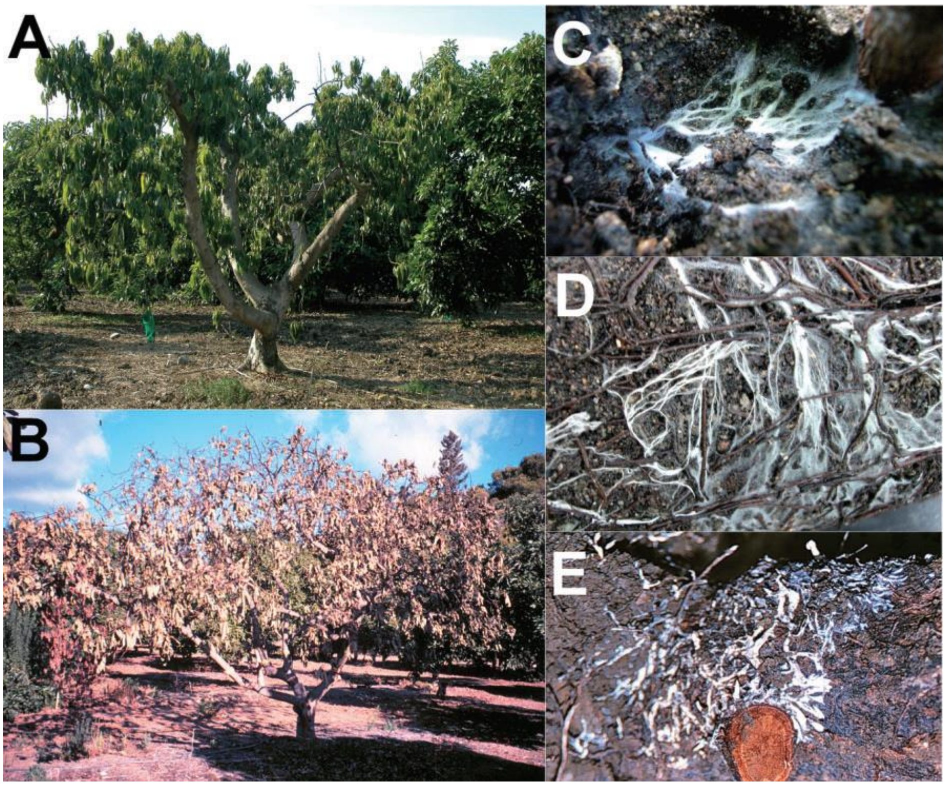

Figure 1.4: The phenotypic and microscopic symptoms caused by *Rosellinia necatrix* on avocado. (A) Healthy 15-year-old avocado plant. (B) Severe aerial symptoms of *R. necatrix* root rot on a 15-year-old avocado plant; Desiccated leaves attached to the wilted plant and overall drying of the plant. (C) The hyphal strands and cords of *R. necatrix* on the soil surface. (D) The hyphal strands and cords of *R. necatrix* spreading from colonized to healthy avocado roots in an avocado plant. (E) *R. necatrix*

spreading on the wood in a 15-year-old avocado plant affected by the pathogen (Pliego et al. 2012).

Pathogenicity and virulence factors of R. necatrix

R. necatrix has pathogenicity factors that allow it to cause WRR in plants (Zumaquero et al. 2019a). The combination of properties including avoidance of host defences, dimorphism and enzymatic activities may serve as virulence mechanisms. During infection, *R. necatrix* remains localised in the roots and absent within the leaves (Arjona-Girona et al. 2017). The presence of toxins (cytochalasin E and rosenecatrone) produced by *R. necatrix* have been shown to contribute to wilting of the leaves (Edwards et al. 2001), and other toxins produced by this pathogen are rosellichalasin (Kimura et al. 1989), rosellinia acid and diketopiperazines (Edwards et al. 2001). These toxins were tested on host seedlings, and they caused black spots, wilting, discolouration, curling, and blackening as well as inhibited germination of rice seedlings (Abe and Kono 1955, Pliego et al. 2012).

Gene expression profiles of *R. necatrix* infecting avocado roots or growing in culture were compared and showed several transcripts related to fungal traits involved in plant cell wall degradation (Laluk and Mengiste 2010), production of toxins and detoxification of those produced by the host or potential effectors (Zumaquero et al. 2019a). The cytochalasin biosynthetic gene cluster was identified, containing 14 genes, within a 36 kb region of *R. necatrix* strain W97 (Shimizu et al. 2018). One gene (putative aflatoxin B1 aldehyde reductase protein) of the putative cytochalasin cluster was up-regulated. At the same time, it was down-regulated in the transcriptomic analyses carried out in the hypovirulent *R. necatrix* strain (Zumaquero et al. 2019a). This gene could play a key role in the virulence of *R. necatrix* on avocado roots. However, the role of the cytochalasin E in virulence remains unclear (Kanematsu et al. 1997).

Fungi can manipulate host cellular structure to suppress defence of the host and facilitate disease progression by releasing effectors (Dodds and Rathjen 2010). Effectors are secreted proteins expressed by plant pathogens to aid infection of specific plant species (Farman and Leong 1998). A recent study on *R. necatrix* infecting susceptible avocado roots demonstrated a total of 23 genes that were predicted to be effectors (Zumaquero et al. 2019a). The predicted effectors showed that 19 encoded for hypothetical proteins and 10 were predicted as apoplastic. One predicted effector demonstrated homology to Lysm1 effector which has been hypothesized to be involved in binding and sequestering chitin oligosaccharides to prevent elicitation of the host immune response or protect fungal hyphae against chitinases secreted by competitors (De Jonge and Thomma 2009). The study suggested that the expression of this effector during *R. necatrix* infection agrees with other studies on overexpression of chitinases in a susceptible avocado-*R. necatrix* interaction (Zumaquero et al. 2019b).

WRR disease control

The incidence of *R. necatrix* in avocado orchards has increased over the years, contributing to yield losses and poor quality of the fruit (Pliego et al. 2012). There are currently challenges in finding potential control strategies for this pathogen. The pathogen survives as a saprophyte in the soil and plant remnants for long periods of time making it capable of infecting new plants in soils which have been cultivated previously (Pliego et al. 2012). This phenomenon has been reported in orchards of subtropical plants planted in soils previously infected with *R. necatrix* (López-Herrera and Zea-Bonilla 2007). It is necessary to do pre-planting treatments, while post-planting treatments must not harm new plants. The most challenging part is controlling the pathogen in the field because of deep-seated infection and deeper penetration of the root system (Sharma et al. 2006). Some fungicides including fluazinam have demonstrated reductions in WRR symptoms on avocado (López-Herrera and Zea-Bonilla 2007, Arjona-López et al. 2020) and biological control of WRR has also drawn attention (Pliego et al. 2011). It is recommended to use an integrated management control strategy to achieve better protection of the plants against WRR.

Cultural control and resistant rootstocks

Cultural control is a practice in plant protection programs which changes the growing conditions making them less favourable for pathogens. This method includes rogueing and burning of diseased plants previously infected with *R. necatrix* (García et al. 2003, Pliego et al. 2012) and periodic testing of soil for the presence of the pathogen, using PCR detection methods (Schena et al. 2002, Schena and Ippolito 2003, Ruano-Rosa et al. 2007). Most importantly, the use of disease-free plant material is important to prevent the spread of the pathogen to previously unaffected areas. At present, no commercial *R. necatrix*-resistant or tolerant rootstock is available (Martínez-Ferri et al. 2016, Zumaquero et al. 2019b). The rootstock BG83 is currently being tested in Spain and demonstrating tolerance against *R. necatrix* (Martínez-Ferri et al. 2016, Zumaquero et al. 2019b).

Physical control

Physical control is a method of getting rid of a pathogen by killing, removing, or setting up barriers that will prevent further infection of the plants. An example of a physical control method that has been used successfully against many pathogens is soil solarization. This process is conducted through wetting of the soil and covering it with a transparent plastic sheet to absorb solar radiation which exposes *R. necatrix* to high temperatures (Pérez-Jiménez 2006). Soil solarization methods in established apple orchards infected with *R. necatrix* have shown to effectively reduce inoculum viability (Sztejnberg et al. 1987). In Spain, avocado orchards infected with *R. necatrix* were exposed to soil solarization and the inoculum load was reduced, but the problem was the re-infection of the pathogen from deeper layers of the soil where the pathogen remained viable, which makes the control of the pathogen using this process expensive (López-Herrera et al. 1999). Another essential factor that hinders eradication of soil pathogens in existing orchards is the low temperatures associated with shaded areas under the tree canopy (López-Herrera et al. 1999). This process also affects beneficial microorganisms in the soil due to the accumulation of high temperatures (López-Herrera et al. 1998). Soil solarization in South Africa has not been effective in established apple orchards and is therefore not recommended (Van Der Merwe and Matthee 1974).

Biological control

Biological control aims to prevent or suppress plant disease development by applying beneficial microorganisms (Heimpel and Mills 2017). Microbial control of plant diseases is a complex process that includes biological control agents (BCAs), the pathogen, and the plant, also the microbiome and plant growth substrate such as the soil (Lugtenberg and Kamilova 2009). The success of BCAs depends on different biological control mechanisms, alone or in combination to suppress plant disease directly or indirectly by competing for space and nutrients, mycoparasitism, antibiosis, mycovirus-mediated cross-protection and induced systemic resistance (Ghorbanpour et al. 2018, Köhl et al. 2019). It is important to consider the mechanisms employed by BCAs to ensure efficiency and sustainability of biological control programs.

To achieve effective control, BCAs should remain active for an extended period under different growth conditions. These requirements are always a challenge, therefore, rendering some BCAs less effective. The demand for biological control has prompted companies to produce plant protection products that contain living organisms (Köhl et al. 2019). These products are applied intensively, and they cause a reduction of disease symptoms or prevent disease occurrence in plants. Limitations of biological products are that, they are less effective and less consistent than chemical products. Understanding the mechanisms of biological control and selection methods for active strains against specific pathogens is needed (Lugtenberg and Kamilova 2009). Biological control against *R. necatrix* in avocado has been conducted in Spain using *Trichoderma* (Freeman et al. 1986, Rosa and Herrera 2009) and *Bacillus* species (González-Sánchez et al. 2013), they demonstrated inhibition effects against the pathogen.

Trichoderma *species*

Trichoderma is a genus of fungi in the family Hypocreaceae, where they are the most prevalent culturable fungi (Vitti et al. 2016). They are the most common BCAs because of their ability to suppress growth of many pathogens (Benítez et al. 2004, Harman 2006). The success of *Trichoderma* spp. is influenced by their capacity to induce plant defence responses, with beneficial effects on plant growth and development (Harman et al. 2004), to improve photosynthetic efficiency, and reprogramming plant gene expression (Shoresh et al. 2010). These fungi are ubiquitous across ecosystems and in all soil types and climatic conditions (Gal-Hemed et al. 2011, Montero-Barrientos et al. 2011, Mukherjee et al. 2013, Waghunde et al. 2016).

Several *Trichoderma* isolates obtained from avocado roots were screened for their antagonistic effect against *R. necatrix* both *in vitro* and *in vivo* (Ruano-Rosa et al. 2003). Most of the *Trichoderma* isolates from avocado roots were characterised and identified as *Trichoderma atroviride*, *Trichoderma virens*, *Trichoderma harzianum* and *Trichoderma cerium* (Ruano-Rosa 2006, Ruano-Rosa et al. 2010). *Trichoderma* spp. have shown protective effects against WRR and gave better results when combined (Rosa and Herrera 2009, Ruano-Rosa et al. 2014b). A study of *Trichoderma harzianum* T22 was investigated as a new approach for control of viruses in tomato plants because chemical treatments were not effective against cucumber mosaic virus (CMV) (Vitti et al. 2016). The *Trichoderma* isolate showed a reduction in CMV symptoms and prevented a reduction in green photosynthetic tissues (Vitti et al. 2016). However, products of *Trichoderma* spp. that are available for commercial purposes to farmers have not been studied extensively against WRR disease in avocado orchards in South Africa.

Bacillus *species*

Bacillus is a genus of gram-positive, rod-shaped bacteria, within the phylum Firmicutes, with 266 named species (Mckillip 2000). Several *Bacillus* species used for control against plant diseases include *Bacillus thuringiensis, Brevibacillus laterosporus, Bacillus subtilis,* and *Bacillus amyloliquefaciens* because of their antagonistic and inhibitory effect (Ruano-Rosa et al. 2014a). The use of these rhizobacterial strains is generally conducted in addition to an existing plant disease control program (Lugtenberg and Kamilova 2009). *Bacillus* and *Pseudomonas* spp. are widely adapted to grow in different environmental conditions, including soil and plant roots (Weller 2007, Earl et al. 2008). These two bacterial strains have been tested against WRR both *in vitro* and *in vivo* (Pliego et al. 2012).

Proper set-up of *in vitro* and *in vivo* trials is important to ensure the selection of potential bacterial BCAs (Pliego et al. 2011). In this regard, repeated applications of *B. subtilis* CB115 and *Pseudomonas chlororaphis* PCL1606 resulted in WRR disease suppression under greenhouse conditions (González-Sánchez et al. 2013). Another greenhouse trial reported that bacterial strains of *P. chlororaphis* PCL1606 and *B.*

subtilis PCL1608 reduced WRR disease severity in avocado plants (Ruano-Rosa et al. 2014b). These bacterial strains demonstrated the ability to control WRR and delayed the appearance of visible aerial symptoms relative to infected control plants (Pliego et al. 2007, Pliego et al. 2012). Products of bacterial strains that are commercially available to farmers have not been studied extensively under South Africa conditions in avocado orchards and greenhouse trials against *R. necatrix*.

Chemical control

The application of chemicals to control plant disease caused by root pathogens is typically injected directly into the soil, the plant or applied through an irrigation system. The use of chemicals contributes to environmental health hazards. Despite the negative impacts, this method of control has been used for centuries worldwide. The most destructive root rot disease of avocado, Phytophthora Root Rot (PRR) is controlled using phosphite treatments (Hardham 2005). However, the mode of action is still uncertain but appears to be direct inhibition of pathogen growth and promoting plant defence through the production of elicitors or reducing pathogen suppressors of plant defence response (Jackson et al. 2000). This product is a systemic fungicide which is translocated in both the xylem and phloem (Guest and Grant 1991) which makes it impossible to work against *R. necatrix* which requires a contact fungicide (Arjona-López et al. 2020). Several fungicides have been tested against WRR, which include fluazinam, carbendazim and thiophanate methyl (López-Herrera and Zea-Bonilla 2007). However, fluazinam showed greater inhibition of *R. necatrix* in both *in vitro* and *in vivo* trials (López-Herrera and Zea-Bonilla 2007).

Fluazinam

Fluazinam is a broad-spectrum fungicide regularly applied in the soil, given its protective systematic or curative effects against many pathogens (Sugimoto 2002). This fungicide acts by inhibiting germinating spores and development of infection structures. The movement and persistence of the fluazinam in soils of planted apples have been investigated previously and was found to be concentrated in the upper soil layers and the concentration decreased with depth (Sharma and Gupta 1985). Another study showed that fluazinam was found at 0.09 µg/mL at a depth of >0.25 m when it suppressed *Monosporascus cannonballus*, the causal agent of sudden wilt of melons (*Cucumis melo var. cantalipensis*) (Cohen et al. 1999). Therefore, these studies suggest that injection of fluazinam at a depth of 0.3 m is essential for effective pathogen control. Since fluazinam is a non-systemic fungicide, it must encounter *R. necatrix* mycelia, a pathogen that survives mostly in the upper soil layers (0.3 m depth) (López-Herrera et al. 1998, López-Herrera et al. 1999).

The efficacy of fluazinam against *R. necatrix* has been compared to carbendazim and thiophanate methyl both *in vitro* and *in vivo* (López-Herrera and Zea-Bonilla 2007*)*. The study showed that fluazinam successfully suppressed *R. necatrix* in both trials, even at 0.1 µg/mL when compared with the other chemicals using the same concentration. The fungicide was further tested in combination with *T. harzianum* and was effective at 0.01 µg/mL against WRR in avocado plants (Ruano-Rosa et al. 2017).

A novel study was conducted on the most effective method of fluazinam application against WRR disease and suggested wetting of the soil (drenching) using an irrigation system is an efficient method (Sugimoto 2002). Recently, a soil injector reaching a depth of 0.3 m was used to apply fluazinam with 24 injection points around each infected avocado plant (Arjona-López et al. 2020). However, the number of applications was lower than the treatments carried out in apple plants in Japan (Sugimoto 2002) but higher than those done in Australia (Stephens 2003). The fungicide was applied in the upper layer of the soil (0.3 m depth) because it has limited movement in the soil since it is an emulsion and aqueous solution. The fungicide was found at the highest concentration in the upper soil layer (Arjona-López et al. 2020). The results from this study showed a significant decrease in *R. necatrix* inoculum load in treated soil compared to non-treated soil from different infected plants (Arjona-López et al. 2020).

Chloropicrin

Chloropicrin is a soil fumigant used to control soil-borne pathogens (Enebak et al. 1990, Ajwa et al. 2010). The effect of chloropicrin has been observed to reduce pathogen load in soil infected with *Rhizobacteria*, *Fusarium*, *Sclerotinia*, *Pythium*, *Verticillium* and *Phytophthora* species (Dangi et al. 2015). The repeated application of the soil fumigant had no cumulative adverse effects over 5 years. However, suppression of nutrients happens in the first few weeks after treatment due to accumulation of ammonia (Li et al. 2017). Increased plant growth was observed following soil fumigation with chloropicrin and a decrease in the accumulation of ammonia (Yamamoto et al. 2008). Plant nutrients increased in the soil solution in days or weeks following soil fumigation with chloropicrin (Massicotte et al. 1998). The flush of nutrients and changes in the biological structure of the soil is a significant benefit to chloropicrin usage (Li et al. 2017).

Chloropicrin degrades without leaching, and no residues were found in the soil or plants (Lembright 1990). Research has been done to understand the rate of degradation of chloropicrin in the soil and it was mostly noted to be within 5 days depending on the soil type, moisture, temperature, and other abiotic factors limiting microbial activity (Noling et al. 2006). The degradation process is important because chloropicrin is converted from active form to basic compounds in the soil which allows the development of soil microbial community to improve soil health.

In South Africa, chloropicrin and 1,3 dichloropropene are currently the most used fumigants for the control of apple replant disease (ARD) (Mazzola and Manici 2012, Cabrera et al. 2015, Nyoni et al. 2019). The complex composition of ARD causative agents makes pre-plant soil fumigation the primary control measure for the disease. Several soil-borne pathogens which include *Pythium*, *Fusarium*, and *Cylindrocarpon* species are proposed as candidate casual agents for ARD (Mazzola and Manici 2012, Nyoni et al. 2019). Also, the presence of root lesion nematodes and the lack of beneficial organisms are believed to aggravate the effects of the plant pathogens. Chloropicrin has fungicidal activity against fungal pathogens and 1,3-dichloropropene targets nematodes (Cabrera et al. 2015). There is no published information on the control of *R. necatrix* in avocado orchards using chloropicrin in South Africa.

Plant physiological response to *R. necatrix*

Changes in plant water relations and biomass (dry mass) production

Root pathogen infections in woody plants cause changes in water relations and limit photosynthesis (Fleischmann et al. 2005, Clemenz et al. 2008, Martínez-Ferri et al. 2016). *R. necatrix* invades the avocado roots and blocks the xylems vessels, causing necrotic and dead tissues of the roots and therefore limits water uptake (Pliego et al. 2009). The earliest response of plants when infected with *R. necatrix* or any pathogen attack is stomatal closure. As consequences of stomatal closure, net CO_2 assimilation (A_N) declines because there is less intercellular CO_2 (C_i) present in the leaves of stressed plants, therefore reduced CO_2 fixation (Da Silva et al. 2008). This phenomenon causes a decrease in dry mass production and allocation, which reduces plant growth and development. The dry mass parameter is a functional response when plants are exposed to stress conditions (James et al. 2005). The presence of a pathogen causes rotting of the roots and therefore more dry mass is expected to be allocated to the roots for generation of new roots (Yin et al. 2005).

The effects of WRR on photosynthesis and gas exchange

Photosynthesis is an important process in plants where light energy is converted to chemical energy in the presence of carbon dioxide (CO_2) and water (H_2O) (Demmig-Adams et al. 2017). Gas exchange is a diffusion of gases from an area of higher concentration to an area of lower concentration, especially the exchange of oxygen (O_2) and CO_2 between an organism and its environment. In plants, gas exchange takes place during photosynthesis and, is determined by A_N and stomatal conductance (g_s) at the leaf level of the plant (Sharkey 2016). The A_N is important in the conversion of CO_2 to energy for plants and g_s measures the amount of CO_2 entering the leaf through stomata. Both A_N and g_s determines the intrinsic water use efficiency (iWUE) of plants. iWUE is the ratio of net photosynthesis and conductance of water vapour, which determines the cost of assimilation per unit of water (Acosta-Rangel et al. 2018). The photosynthetic rates determine the status of the plants (Demmig-Adams et al. 2017) and their ability to survive under water or pathogen stress conditions. The immediate shutdown of stomata following water limitation reduces CO_2 diffusion into the leaf and subsequently, a decrease in A_N is experienced by plants (Lawlor 1995). This effect is further explained by a lowered intercellular CO_2 concentration that results in limitation of photosynthesis at the acceptor site of Rubisco (Ghashghaie et al. 1992) or by the direct inhibition of photosynthetic enzymes (Haupt-Herting and Fock 2000) or ATP synthase (Tezara et al. 1999, Nogués and Baker 2000).

The effect of *R. necatrix* caused an early decline in A_N and g_s at 22 days post-infection (dpi) in 'Dusa®' plants (Zumaquero et al. 2019b). The first reductions before the appearance of aerial symptoms could be a result of toxins which are sent to the leaves at early stages of *R. necatrix* infection (Martínez-Ferri et al. 2016). However, stomatal limitation appeared to be the main factor causing a decrease in A_N and g_s in infected plants (Zumaquero et al. 2019b). The presence of *R. necatrix* on the roots of avocado resulted in a decrease in water uptake, and plants responded by closing their stomata to maintain their leaf water status (Davies and Flore 1986). The changes in root permeability and conductivity in the presence of the pathogen further affected water uptake in plants (Jackson 2004, Else et al. 2008). The iWUE was reduced in 'Dusa®' plants compared to BG83 tolerant plants when infected with *R. necatrix* (Zumaquero et al. 2019b). The decrease in this parameter was associated with a decline in both A_N and g_s in 'Dusa®' plants. The importance of iWUE is to determine plant growth in water stress conditions (Liu and Stützel 2004). *R. necatrix*-tolerant BG83 plants demonstrated no significant decline in gas exchange variables when infected with *R. necatrix* (Zumaquero et al. 2019b). BG83 plants showed the ability to reduce the pathogen's effect on water limitation in the plant and avoiding A_N decline (Zumaquero et al. 2019b). The tolerance of BG83 to *R. necatrix* is related to the ability to induce protease inhibitors and their negative regulators, including genes related to tolerance of salt and osmotic stress (Zumaquero et al. 2019b) In this regard, studies are revealing the mechanisms employed by BG83 against *R. necatrix* relative to susceptible rootstocks (Zumaquero et al. 2019a, Zumaquero et al. 2019b).

The effect of WRR on chlorophyll fluorescence

Chlorophyll fluorescence is the re-emitted light by chlorophyll molecules during the return from excited to non-excited states (Baker 2008). The light absorbed by a leaf of a plant undergoes three fates; (1) use in photosystem II (PSII) in the process of photochemistry, (2) dissipated as heat and or (3) re-emitted as fluorescence (Maxwell and Johnson 2000, Baker 2008). The goal of understanding chlorophyll fluorescence in the process of photosynthesis is because it provides information about the state of PSII in leaves and energy absorbed by chlorophyll molecules (Maxwell and Johnson 2000). The PSII is the vulnerable part in the thylakoid membrane (site of light-dependent reactions of photosynthesis) and the first to be affected by stress in plants (Maxwell and Johnson 2000, Baker 2008, Granum et al. 2015).

Maximum photochemical efficiency of photosystem II (F_v/F_m ratio) is important in quantifying the amount of light energy travelling through the fluorescence pathway and an excellent indicator of stress in photosynthesizing eukaryotic cells (Buschmann and Lichtenthaler 1998, Baker 2008, Rolfe and Scholes 2010). The F_v/F_m ratio for a healthy plant ranges from 0.75 to 0.85 depending on the age and health of the leaf (Maxwell and Johnson 2000, Abdel-Fattah et al. 2007, Baker 2008). A previous study demonstrated that the F_v/F_m ratio decreased when the first WRR symptoms appeared at 28 dpi in susceptible avocado plants (Granum et al. 2015). This decrease was assumed to be caused by oxidative damage of PSII, due to water stress caused by WRR. However, usually as the consequence of stomatal closure following water stress, the F_v/F_m ratio demonstrates lower values due to limitation of CO_2 inside the leaf and lack of enough CO_2 fixation which results in photo-inhibition (Flexas et al. 2002, Baker 2008). Another study showed that 'Dusa®' plants had early reductions in steady-state yield fluorescence normalized to initial minimal fluorescence (F_s/F_o) before the development of WRR symptoms (Martínez-Ferri et al. 2016). In contrast, tolerant BG83 plants showed no variation when infected with *R. necatrix* (Martínez-Ferri et al. 2016). The F_s/F_o is an excellent indicator of decreasing A_N, g_s and non-photochemical quenching (NPQ) during mild water stress, which provides a useful tool for early detection of water stress (Flexas et al. 2002).

The continued decrease in F_s/F_o is related to an increase in NPQ under non-limiting water conditions (Martínez-Ferri et al. 2016). NPQ is a process whereby plants employ a mechanism to protect themselves from excess light energy (Maxwell and Johnson 2000, Baker 2008). A previous study demonstrated that partially resistant 'Dusa®' and 'Duke 7' plants infected with *P. cinnamomi* and flooded showed an increase in NPQ values indicating stress at the end of the trial (Reeksting et al. 2014). This suggested that the increase in NPQ was protection through xanthophyll cycling (Müller et al. 2001). Xanthophyll cycling is a mechanism that protects plants against oxidative stress (Latowski et al. 2011). 'Dusa®' and 'Duke 7' plants further showed lower values of photochemical quenching (qP) (Reeksting et al. 2014). qP is the proportion of light absorbed by chlorophyll associated with PSII that is used in photochemistry (Maxwell and Johnson 2000). Therefore, when the amount of light used for photochemistry is reduced, NPQ and qP change. In a previous study, the change in qP was associated with efficiency of photosystem II (Φ_{PSII}) as shown by the lower values observed in 'Duke 7' and 'Dusa®' plants when exposed to flooding and *P. cinnamomi* stress (Reeksting et al. 2014). The Φ_{PSII} was reduced because light energy was no longer transported away from PSII, and the activation of enzymes was affected because of the shutdown of the reaction centres. The Φ_{PSII} is the quantum yield of PSII photochemistry (Maxwell and Johnson 2000).

Conclusion

R. necatrix is a fungal soil-borne pathogen that causes WRR in many woody plants and has become important globally. Researchers are interested in how this pathogen causes WRR disease and spreads from infected plants to healthy plants. In particular, the pathogen has spread significantly because many control strategies have failed to completely control WRR and tolerant commercial rootstocks are still not available. In Spain, BG83 is a promising tolerant rootstock that can be used against the pathogen. Understanding of the avocado-*R. necatrix* interaction is important to establish tools that will assist in early detection of the pathogen before WRR symptoms are evident. In this way, physiological responses have gained interest in recent years as a tool to analyse the effect of pathogens in combination with molecular techniques. The processes of photosynthesis and chlorophyll fluorescence measurements are now important to analyse plants under stress conditions in a greenhouse or controlled environment. The lack of control products that can completely eradicate the presence of *R. necatrix* pose a threat to the avocado industry. Therefore, knowledge on the use of chemical and biological control against *R. necatrix* will improve the integrated management approach in avocado orchards and nurseries.

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CHAPTER 2

The effects of *Rosellinia necatrix* on avocado physiology

Abstract

Rosellinia necatrix Berl. ex Prill. is the causal agent of White Root Rot (WRR), a fatal disease which affects many woody plants including avocado (*Persea americana* Mill). This pathogen was recently discovered in avocado orchards in South Africa for the first time in 2016, potentially threatening the commercial industry that is currently using rootstocks partially resistant to *Phytophthora cinnamomi* for sustainable production in the presence of Phytophthora Root Rot (PRR). Early detection and rapid responses are important to avoid the spread of *R. necatrix*. However, above-ground symptoms are often confused with those of PRR and molecular detection methods are limited to producers who have access to intrinsic sampling procedures and molecular laboratory assays. The current study therefore aimed to assess the physiological response of avocado during *R. necatrix* infection, before the first appearance of White Root Rot (WRR) disease symptoms in three avocado rootstocks to serve as an early warning. Both leaves and roots were assessed for WRR based on a visual symptom scoring system. The first wilting symptoms appeared at 35 days-post infection (dpi) in all three rootstocks examined. Infected plants showed significantly lower values of stomatal conductance and net CO_2 assimilation when the first visible stress symptoms manifested as wilting leaves were evident relative to control plants. The maximum quantum efficiency of photosystem II significantly decreased to 0.70 as compared to values above 0.75 for the controls of all the infected rootstocks as early as 28 dpi prior to development of wilt symptoms. The early detection of stress in infected plants before the development of visual symptoms will prompt further investigation using PCR confirmation assays to minimize the time between infection and exposure to the pathogen and implementing control measures. Early detection and rapid response for control of WRR are necessary to limit the spread of the disease.

Keywords: Chlorophyll fluorescence, Pathogen, Photosynthesis, Stomatal conductance, Rootstocks.

Introduction

Avocado (*Persea americana* Mill), a member of the Lauraceae family, is an important fruit crop worldwide (Zumaquero et al. 2019b) and its consumption has increased over the last decade. To meet this challenge, supply has had to increase, through increasing areas planted to avocado and preventing losses as a result of diseases. Two of the most important avocado root diseases are Phytophthora Root Rot (PRR) and White Root Rot (WRR) (Zumaquero et al. 2019b). *Phytophthora cinnamomi* Rands, the causal agent of PRR, affects avocado production worldwide (Hardham 2005), while, *Rosellinia necatrix* Berl. ex. Prill, the causal agent of WRR, is most prominent in temperate and subtropical climates (Pliego et al. 2012, Kondo et al. 2013). *P. cinnamomi* has a wide host range of 5000 plant species (Hardham 2005) and development is promoted in areas with high rainfall or where water is not limiting, while *R. necatrix* has a wide host range of 475 plant species (Farr and Rossman 2020). The pathogen has caused losses in avocado production in Spain and Israel (Pliego et al. 2012). In South Africa, the first detection of WRR in avocado orchards occurred in September 2016, where several plants in a commercial orchard died due to the disease (van den Berg et al. 2018).

Detection of *R. necatrix* relies mostly on visual symptom detection followed by PCR confirmation assays (Schena and Ippolito 2003, Ruano-Rosa et al. 2007). This strategy is time-consuming, expensive, and human resources are required for sampling, processing, and laboratory assays. This make it difficult to sample all the trees in a commercial orchard before symptom development. However, if a plant is suspected of WRR infection, PCR is used as an early confirmation tool for pre-symptomatic stage infection. PCR accuracy depends on the selection of infected plant material for the assay, which can be scarce in newly infected plants. A consequence of late detection of the pathogen is that infected plants become inoculum sources for further spread of WRR to surrounding plants. This suggests that PCR, in some cases, is impractical and expensive for the early detection of WRR, especially in large orchards. Recent studies have explored the possibility of measuring physiological responses of avocado plants infected with *R. necatrix* under greenhouse conditions

(Martínez-Ferri et al. 2016, Zumaquero et al. 2019b). This strategy of investigation facilitates the use of pre-symptomatic screening tools for stressed plants in the field.

R. necatrix infection damages plant roots, and as a result, the uptake of water and nutrients is limited. One of the first responses is therefore stomatal closure, followed by leaf wilting (Fleischmann et al. 2004, Pliego et al. 2009). Plants close their stomata under water stress, resulting in a decline in stomatal conductance (g_s) which may lead to a decrease in CO_2 availability and CO_2 assimilation (A_N) (Vu 1999, Medina et al. 2002, Machado et al. 2005). These changes are related to reductions in mesophyll CO_2 conductance (Vu 1999) and in the carboxylation efficiency of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) enzyme (Jifon and Syvertsen 2003). An example of the effect of root disease on leaf physiological processes occurred in 'Duke 7' plants infected with *P. cinnamomi*, where stomatal limitations to A_N were due to decreases in g_s (Reeksting et al. 2014). A decrease in A_N and g_s appeared at 22 days post-infection (dpi) in infected *P. cinnamomi* partially resistant 'Dusa®' plants (Reeksting et al. 2014). A similar response was observed at 22 dpi for 'Dusa®' plants infected with *R. necatrix* which demonstrated a decrease in A_N and g_s and preceded visible WRR symptoms (Zumaquero et al. 2019b). This suggests that stomatal limitations to A_N occurred at an early stage of WRR infection and demonstrated high susceptibility of 'Dusa®' plants to the pathogen. In contrast, no significant variation in gas exchange parameters were observed as WRR disease progressed in the tolerant BG83 plants (Zumaquero et al. 2019b).

WRR development caused a decrease in A_N at the photochemical level in 'Dusa®' plants (Martínez-Ferri et al. 2016). The early and significant reduction in the steady-state yield of fluorescence normalized to initial minimal fluorescence (F_s/F_o) appeared before the first aerial WRR symptoms became evident (Martínez-Ferri et al. 2016). Previous studies have demonstrated that F_s/F_o is an excellent indicator for decreasing g_s, and non-photochemical quenching (NPQ) during mild water stress (Flexas et al. 2002). In contrast, the maximum quantum efficiency of photosystem II (F_v/F_m ratio) in plants infected with *P. cinnamomi* at early stages of infection did not show a significant decrease when quite severe symptoms were apparent during the trial (Reeksting et al. 2014). Also, the reductions in the efficiency of photosystem II (Φ_{PSII}) were accompanied by a reduction in photochemical quenching (qP) and increase in NPQ in ‘Dusa®’ and ‘Duke 7’ plants infected with *P. cinnamomi* (Reeksting et al. 2014).

This study aimed to investigate physiological parameters, including leaf gas exchange and chlorophyll fluorescence, to determine the timing of the onset of stress in plants following infection with *R. necatrix*. The study hypothesised that *R. necatrix* infection would cause stomatal closure resulting in a reduction in photosynthesis and therefore a decline in dry mass production relative to uninfected control plants. Furthermore, it was hypothesised that avocado plants infected with *R. necatrix* would demonstrate elevated stress levels as indicated by reduced F_v/F_m ratio before visible WRR symptoms were apparent, due to reduced photosynthetic ability and intercellular CO_2 concentration.

Materials and methods

Plant material

Twelve-month-old clonal avocado rootstocks ('Dusa®', R0.38 and R0.06), with no grafted scion, obtained from Westfalia Technological Services (WTS), Tzaneen, South Africa, were transplanted into 5 L plastic bags containing a soil-perlite mixture (mixed at 1:1 ratio) and placed in a greenhouse on the experimental farm of the University of Pretoria, Pretoria, South Africa (25° 47' 7.38" S 28° 30.44" E). 'Dusa®' was selected for this trial because is an industry rootstock. The rootstocks, R0.38 and R0.06 were selected based on their performance against *Phytophthora cinnamomi*. The soil was sterilized before planting using an electrode boiler solution (Marshall-Fowler, Randfontein, South Africa). A micro-sprinkler irrigation system watered the plants with an application rate of 20 L/h for 30 min twice a day at an interval of 12 h. Nitrosol (Pest and Chemical Supply, South Africa) was used as a nutrient supplement, the solution was prepared using 50 mL of the product in 5 L of water and the application rate per plant was 50 mL of the solution once every 14 days.

Greenhouse conditions

Weather variables were recorded by a Campbell Scientific automatic weather station situated in the open, approximately 300 m from the greenhouse. The average daily maximum temperature during the trial period was 25.7°C, with an average daily minimum temperature of 11.1°C. Average incoming solar radiation during the trial period was 22.31 MJ $m^{-2}day^{-1}$ and ranged from 6.41 to 31.84 MJ $m^{-2}day^{-1}$. The photosynthetically active radiation (PAR) in the greenhouse throughout the trial was on average 299.81 µmol $m^{-2}s^{-1}$, with a maximum of 889.17 µmol $m^{-2}s^{-1}$.

R. necatrix strain and infection strategy

A virulent strain of *R. necatrix* (ARP-2017-Rn2) (van den Berg et al. 2018), previously isolated from avocado in South Africa, was obtained from the culture collection of the Avocado Research Programme (ARP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. The isolate was grown on $\frac{1}{2}$ potato dextrose agar (PDA) at a concentration of 19.5 g/L (BioLab, South Africa) at 25°C in the dark for 7 days before preparation of the fungal inoculum.

Fungal inoculum was prepared following a modified method of Negishi et al. (2011). Wooden chopsticks were cut into 2-cm pieces, washed, soaked in distilled water for 2 days and autoclaved at 120°C for 15 min, followed by drying at 65°C for 36 h. The chopsticks were then autoclaved again for another 15 min. The sticks were aseptically placed in sterile Petri dishes (four per plate) containing 20 mL of $\frac{1}{2}$ PDA inoculated in the centre with a 5-mm disc of *R. necatrix* mycelia. The cultures were incubated at 25°C in the dark upright and inverted after 24 h. They were ready for infection of avocado plants when mycelia completely colonized all the chopsticks in the Petri plates. In order to infect the avocado plants, a piece of colonized chopstick was placed in each of the four holes prepared 2 cm away from the root collar. For the control plants, sterile chopsticks were used.

The effect of WRR disease development in avocado plants

The health of 10 avocado plants was assessed by monitoring aerial disease development weekly (Table 2.1) and evaluating the roots at 56 dpi (Table 2.2). The scores for both aerial and root WRR symptoms were used to calculate the disease severity as presented in Equation 2.1.

Table 2.1: The scoring system for avocado aerial plant parts following *Rosellinia necatrix* infection (González-Sánchez et al. 2013).

Criteria	Disease scoring
No leaves wilting	0
Drooping of leaves	1
Number of leaves dropped	2
More than 50% leaves wilted	3
Most leaves wilted (>50%)	4
All leaves wilted and collapsed	5
Overall drying of plant	6
Dead plant	7

Table 2.2: The scoring system for root symptoms of avocado plants following *Rosellinia necatrix* infection (González-Sánchez et al. 2013).

Criteria	**Disease scoring**
All healthy	0
Mostly healthy-some necrosis (10%)	1
50% of roots necrotic	2
More than 50% of the roots necrotic.	3
All roots necrotic	4

Disease severity was calculated for the roots and the leaves using the formula by Sherwood and Hagedorn (1958).

$$\text{Percent Disease Index} = \frac{\text{Sum of all disease ratings x 100\%}}{\text{Total number of ratings x maximum disease grade}} \qquad (2.1)$$

The effect of WRR on the biomass (dry mass) in avocado plants

The dry mass of roots, stems and leaves were determined for each plant after oven drying at 70°C for 3 days for both infected and control plants of all three rootstocks. These measurements were done for 10 plants of each rootstock for both control and infected plants. When the trial ended, the number of green leaves attached to the plants were counted, and the stem length was measured. The root:shoot ratio (R:S) was used to assess the overall health of the plants (Wu et al. 2008) as presented in Equation 2.2. Lastly, relative water content (RWC) was determined, as it is an appropriate estimate of cellular hydration, leaf water deficit and physiological water status of plants (Sharon et al. 2001) as presented in Equation 2.3.

$$\text{Root: shoot ratio} = \frac{\text{Root dry weight}}{\text{Aboveground plant parts dry weight}} \qquad (2.2)$$

$$\text{Relative water content} = \frac{\text{Fresh weight} - \text{dry weight}}{\text{Fresh weight}} \qquad (2.3)$$

Detection and identification of *R. necatrix* in avocado roots

Root material was harvested from the plants at 56 dpi for *R. necatrix* confirmation assays. The soil from the roots was removed using tap water in all the treatments Fresh samples of root material from control and infected plants of all the rootstocks were also surface sterilized with 70% ethanol, washed in distilled water, and dried in a laminar flow hood. The root samples were transferred to $\frac{1}{2}$ PDA and incubated in the dark at 25°C for a day before the plates were inverted and incubated for an additional 6 days. The cultures were visualized using light microscopy for pear-shaped swellings adjacent to septa, which is a distinctive structure of *R. necatrix* (Pliego et al. 2012). In addition to primary isolation, approximately 30 mg of ground root material was used to extract DNA using a modified CTAB protocol (Engelbrecht et al. 2013) for molecular detection. A species-specific fragment (~500 bp) was amplified from DNA of each sample using primers R3 (Primer forward (5' - 3') CGA AGTGCC CTA CCC TGT TA) and R8 (Primer reverse (5' - 3') CCG AGG TCA ACCTTT GGT ATA G) (Schena et al. 2002), to confirm the presence and identify the pathogen. The PCR products were separated on a 1.5% agarose gel by electrophoresis at 90 V for 45 min.

Leaf gas exchange and chlorophyll fluorescence measurements

The plants were assessed for photosynthetic performance throughout the trial by measuring leaf gas exchange [net CO_2 assimilation (A_N) and stomatal conductance (g_s)] in avocado plants, The measurements were recorded weekly on five replicates per treatment (control and infected plants of the three rootstocks) from mid-morning until afternoon (10:00 – 14:00 h) on the first fully expanded mature leaves from the top of each plant. Once selected these leaves were labelled for accurate repeated measurement for the duration of the experiment. The measurements were performed using an open-path portable photosynthesis system LI-6400XT (LI-COR, Lincoln, Nebraska, USA) equipped with 2 x 3-cm LED light source (LI-COR, Lincoln, Nebraska, USA) and a CO_2 mixer (6400-01) to modify the incoming air CO_2 concentrations. The operating flow rate was 500 mL min^{-1} and the CO_2 partial pressure was 400 µmol CO_2 mol^{-1} air during the measurements of leaf gas exchange parameters. Humidity within the sample chamber ranged from 50 to 70% over the trial period, and leaf temperature varied between 11°C and 25°C. Using established equations embedded in the

LI6400XT software (Long and Bernacchi 2003), A_N and g_s were estimated, and then intrinsic water use efficiency (iWUE) was calculated as A_N/g_s to determine water use efficiency in control and infected plants of the three rootstocks.

Chlorophyll fluorescence [maximum quantum efficiency of photosystem II (F_v/F_m ratio), the efficiency of photosystem II (Φ_{PSII}), non-photochemical quenching (NPQ) and photochemical quenching (qP)] measurements were performed using the 6400-40 leaf chamber fluorometer (LI-COR, Lincoln, Nebraska, USA). The measurements were taken mid-morning (10:00 – 12:00 h) the day after gas exchange measurements and on the same leaves. The saturation pulse method was used to determine all fluorescence parameters (Schreiber et al. 1986). In order to assess the initial minimal fluorescence (F_o) in the dark or the steady-state fluorescence (F_s) in the light, leaf samples were exposed to a weakly modulated measuring beam. Leaves were covered in aluminium foil for 30 min to dark adapt the leaves before the measurement. A saturation flash of light (12000 µmol quanta $m^{-2}s^{-1}$) was administered for 0.8 s to assess the maximal fluorescence level, either in the dark, when photosystem II (PSII) centres closed (F_m), or under light conditions (F_m'). The leaves were immediately darkened after every saturation pulse and subsequently exposed to far-red light for 5.5 s to determine the minimal fluorescence yield of the pre-illuminated sample (F_o'). The steady-state fluorescence yield (F_s) was normalized to dark-adapted fluorescence yield (F_o). Measurements of F_m and F_o determined the maximal photochemical efficiency of PSII [$F_v/F_m = (F_m-F_o)/F_m$] and non-photochemical fluorescence quenching ($NPQ = (F_m - F_m')/(F_m')$) (Bilger and Björkman 1990, Bilger et al. 2001). The relative quantum yield of PSII photochemistry ($\Phi_{PSII} = (F_m'-F_s)/F_m'$), photochemical quenching $(qP) = (F_m' - F_s)/(F_m'-F_o')$, maximum photochemical efficiency of the open reaction centres of PSII $(F_v' - F_o')/F_m'$ were calculated as outlined by Genty et al. (1990).

A-C_i response curves

Measurements of the leaf net CO_2 assimilation response to increasing intercellular CO_2 concentrations (A-C_i) were performed on the same leaves as those used for leaf gas exchange and chlorophyll fluorescence measurements. The curves were performed under saturating photosynthetic photon flux density (PPFD) of 1000 µmol $m^{-2}s^{-1}$ and were conducted from mid-morning to mid-afternoon (11:00 to 14:00 h). The intercellular CO_2 concentration (C_i) was controlled by varying air CO_2 concentrations (C_a) from 50 to 2000 µmol CO_2 mol^{-1}. The protocol to perform A-C_i response curves followed the recommendations previously described by Long and Bernacchi (2003). The A-C_i response curves were used to estimate the maximum rate of ribulose-1,5-bisphosphate (RuBP) carboxylation (V_{cmax}) and the maximum rate of electron transport driving RuBP regeneration (J_{max}). The Farquhar model of leaf photosynthesis was fitted to the A-C_i curves to calculate V_{cmax} and J_{max} using Microsoft Excel Spreadsheet-based software (Farquhar et al. 1980, Von Caemmerer 2000, Sharkey et al. 2007). The CO_2 compensation point, in the absence of daytime respiration, and the Michaelis-Menten constants of the Rubisco activities for CO_2 and O_2 (Kc and Ko, respectively) were calculated at the measured leaf temperatures according to the temperature-dependent equations and parameters previously described by Bernacchi et al. (2001). Von Caemmerer (2000) recommended that daytime respiration (Rd) should be assumed to be 0.01 of V_{cmax}.

Experimental design and statistical analysis

A minimum of five plants per leaf gas exchange and chlorophyll fluorescence analysis were randomly chosen from 15 plants per treatment for each of the three rootstocks. Dry mass determination and disease severity ratings were mean values of 10 plants per treatment. The mean values of each variable from A-C_i response curves were derived from three replicates per treatment and rootstock. Microsoft Excel and statistical analysis (SAS) 9.4 version were used to identify significant differences between treatments. The results were subjected to analysis of variance (ANOVA) and the means of all treatments were separated using Tukey's test and the Least Significant Difference (LSD) both at a 5% level of significance ($p<0.05$).

Results

Detection and identification of *R. necatrix* on avocado roots

R. necatrix was successfully re-isolated from diseased avocado roots of infected plants of all the rootstocks. No fungal growth on root material isolated from control plants of all the rootstocks was observed (Figure 2.1A). *R. necatrix* was isolated from diseased root material, purified successfully (Figure 2.1B and C) and the identity was confirmed as *R. necatrix* with PCR species-specific primers (R3 and R8) that yielded a product of approximately 500 bp (Figure 2.2).

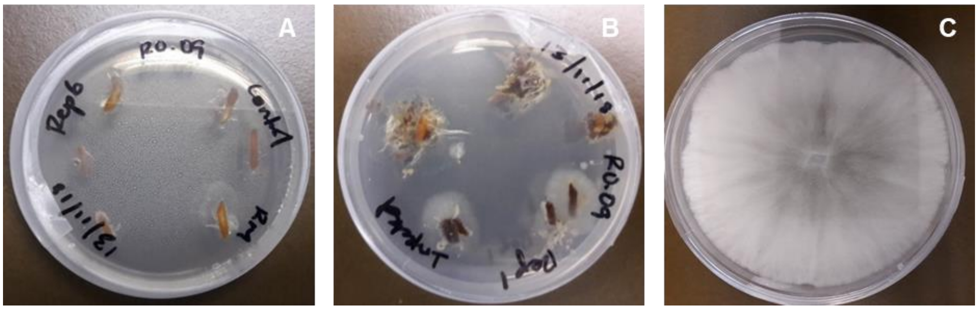

Figure 2.1: The mycelial growth of *Rosellinia necatrix* from infected plant root material. (A) Root material plated out on $\frac{1}{2}$ PDA from control plants and no fungal growth was observed. (B) Root material plated on $\frac{1}{2}$ PDA from infected plants and white mycelia was observed. (C) Purified culture of *R. necatrix* grown from the infected root material.

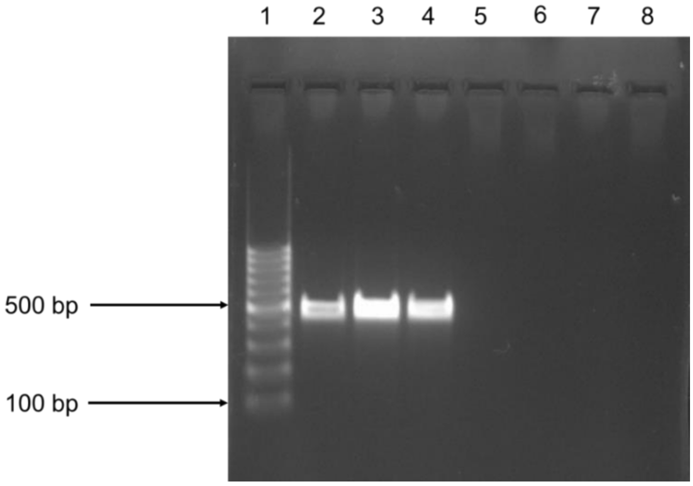

Figure 2.2: PCR confirmation assays of *Rosellinia necatrix* from avocado root material of three rootstocks. The R3 and R8 *R. necatrix* species-specific primers

yielded a product of approximately 500 bp confirming the presence of the pathogen in root samples of infected plants, respectively. 1. 100 bp molecular marker; 2-4 Infected samples; 5-7 control samples (‘Dusa®’, R0.38 and R0.06, respectively) and 8. Non-template control.

WRR symptoms on avocado plants

Control plants demonstrated no signs of stress for all the rootstocks throughout the trial (Figure 2.3A). Typical WRR symptoms, such as abscission and wilting of one or two leaves, were first observed at 35 dpi on infected plants, however, at this stage, more than 50% of the plants were still healthy and no signs of severe stress were observed in any of the rootstocks. As WRR progressed (42 dpi), wilting of leaves of infected plants was pronounced in more than 60% of the plants from all the rootstocks. When the trial ended at 56 dpi, 90% of infected plants from each rootstock had completely wilted (Figure 2.3A). Some infected plants had no green tissue and were desiccated at the end of the trial. After 56 dpi, the disease severity of infected plants of ‘Dusa®’, R0.38 and R0.06 were 94, 90 and 85% respectively, while control plants all showed less than 10% aerial symptoms from leaf drop and drooping of a few leaves but not due to *R. necatrix* since it was not isolated from the plants (Figure 2.3B).

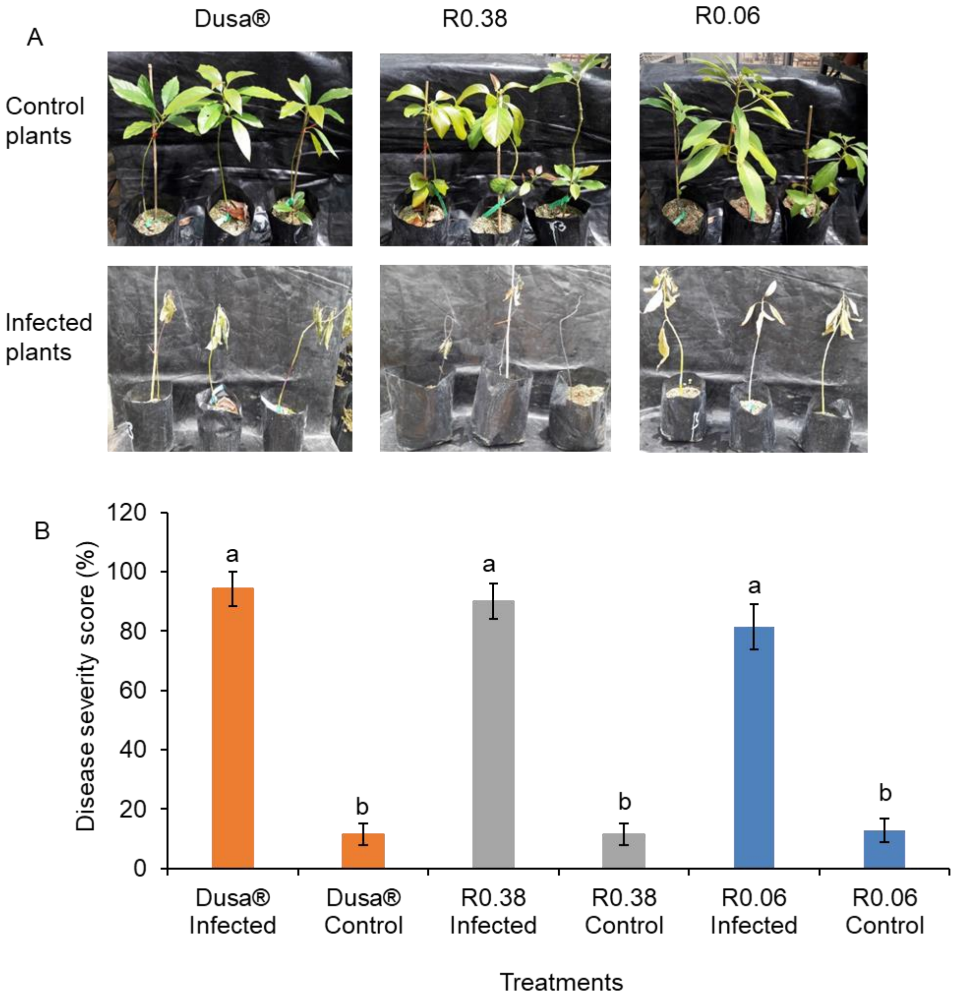

Figure 2.3: Visual assessment of the effect of White Root Rot disease on aerial parts of avocado plants of the three rootstocks. (A) The infected plants demonstrated severe leaf wilting at 56 dpi, while control plants showed healthy phenotypic symptoms. (B) Percentage of disease severity of infected and control plants. Bars indicate standard errors of the mean of 10 replicates and means with the same letter were not significantly different according to Tukey's LSD test (P<0.05).

At 56 dpi, infected plants demonstrated necrotic and dead black roots covered with white mycelia, while control plants had fewer black roots, but these roots were not necrotic (Figure 2.4A). Infected plants of 'Dusa®', R0.38 and R0.06 had root disease severity scores of 92, 90 and 91%, respectively, while control plants of all the rootstocks showed less than 10% (Figure 2.4B). The 10% disease severity on control plants of the roots are not due to the pathogen as demonstrated above since no *R. necatrix* isolated from the control plants. The infected plants were therefore severely affected by WRR when compared to control plants. There was no significant difference between the infected rootstocks tested, and all were equally susceptible to WRR.

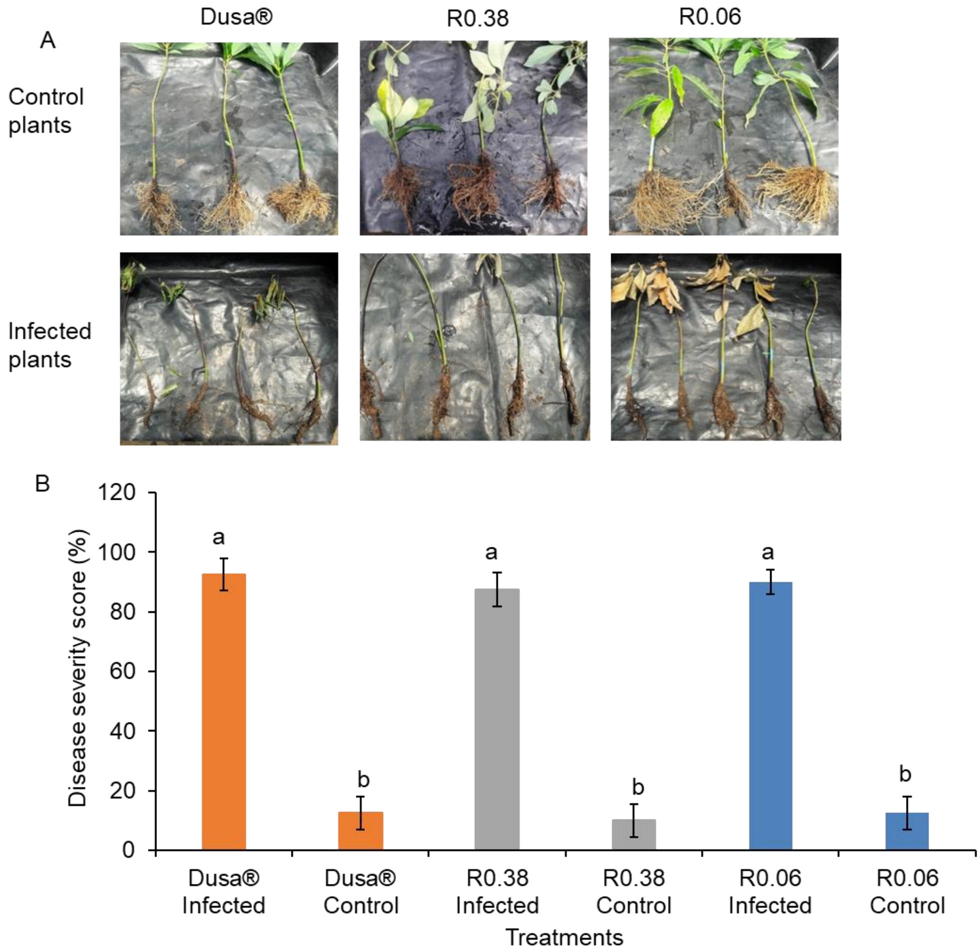

Figure 2.4: Visual assessment of the effect of White Root Rot on the roots of control and infected plants. (A) White mycelial growth and necrotic roots were observed on infected plants at 56 dpi, while control plants had healthy roots. (B) Percentage disease severity of infected and control plant roots. Bars indicate standard errors of the mean of 10 replicates and means with the same letter were not significantly different according to Tukey’s LSD test ($P<0.05$).

The effect of *R. necatrix* on the biomass (dry mass) of avocado plants

R. necatrix had a significant impact on dry mass of infected plants, as compared to control plants of all the rootstocks (Table 2.3). Infected plants had lower leaf, root and stem dry mass compared to control plants. Most of the infected plants had wilted and desiccated when the trial ended, which further contributed to the significant decrease in dry mass when compared to control plants. In addition, stem length and the number of leaves of infected plants were significantly different when compared to control plants of all the rootstocks. An exception was 'Dusa®', where stem length of infected plants was not significantly different to control plants. As WRR progressed, growth and development of the stems appeared to be limited. The reduced number of leaves of infected plants was mostly due to increased leaf abscission at the post-symptomatic stage. Infected and control plants of all the rootstocks were also significantly different when relative water content (RWC) and root:shoot ratio (R:S) were compared. Infected plants had lower values of RWC and R:S in all the rootstocks compared to control plants.

Table 2.3: The effect of *Rosellinia necatrix* on the dry mass of avocado plants. The values are for the mean dry mass of stem, leaf and root tissue, root:shoot ratio (R:S), relative water content (RWC), stem length and the number of leaves of infected and control plants of three rootstocks taken at 56 days post-infection ('Dusa®', R0.06 and R0.38).

	'Dusa®'		**R0.06**		**R0.38**	
	Control	**Infected**	**Control**	**Infected**	**Control**	**Infected**
Leaf (g)	10.92±0.83^{b}	5.19±1.08^{c}	18.83 ±2.4^{a}	5.76±1.60^{c}	15.09±1.58^{a}	6.99±0.62^{c}
Stem (g)	7.33±0.8^{b}	4.05±0.4^{d}	10.80 ±1.1^{a}	5.80±0.7^{cd}	11.28±0.7^{a}	4.20±0.8^{cd}
Root (g)	12.94±1.2^{b}	3.50±0.4^{d}	17.59 ±1.9^{a}	4.19±0.9^{cd}	15.47±1.1^{a}	4.01±0.9^{cd}
Leaf number	46.50±3.5^{b}	15.20±4.3^{c}	62.8 ±3.6^{a}	13.20±4.3^{c}	51.00±3.8^{b}	8.20±1.0^{c}
Stem length	499.00±44^{ab}	392±19^{bc}	567.50±46^{a}	360.40±33^{c}	538.10±34^{a}	346.00±22^{c}
R:S ratio	0.71±0.0^{a}	0.38±0.1^{b}	0.59±0.04^{a}	0.36±0.0^{b}	0.56±0.0^{a}	0.35±0.1^{b}
RWC	0.72±0.01^{a}	0.35±0.04^{b}	0.71±0.01^{a}	0.40±0.09^{b}	0.73±0.01^{a}	0.39±0.04^{b}

Different letters indicate significant differences in the same row between control and infected plants in each rootstock based on a Tukey's LSD statistical test (P<0.05).

Assessment of leaf gas exchange measurements in avocado plants infected with *R. necatrix*

The effect of R. necatrix *on net CO_2 assimilation (A_N) and stomatal conductance (g_s) in avocado plants*

There were no significant differences prior to development of visual symptoms as wilting of leaves of infected plants compared to control plants. As WRR progressed, the decrease in A_N (Figure 2.5A) and g_s (Figure 2.5B) of infected plants became significant at 35 dpi, when the first stress symptoms appeared as wilting of leaves of infected plants of all three rootstocks. Significantly lower A_N and g_s values of infected plants relative to control plants continued until the measurements ended at 49 dpi. All the rootstocks responded similarly throughout the trial for infected plants compared to control plants.

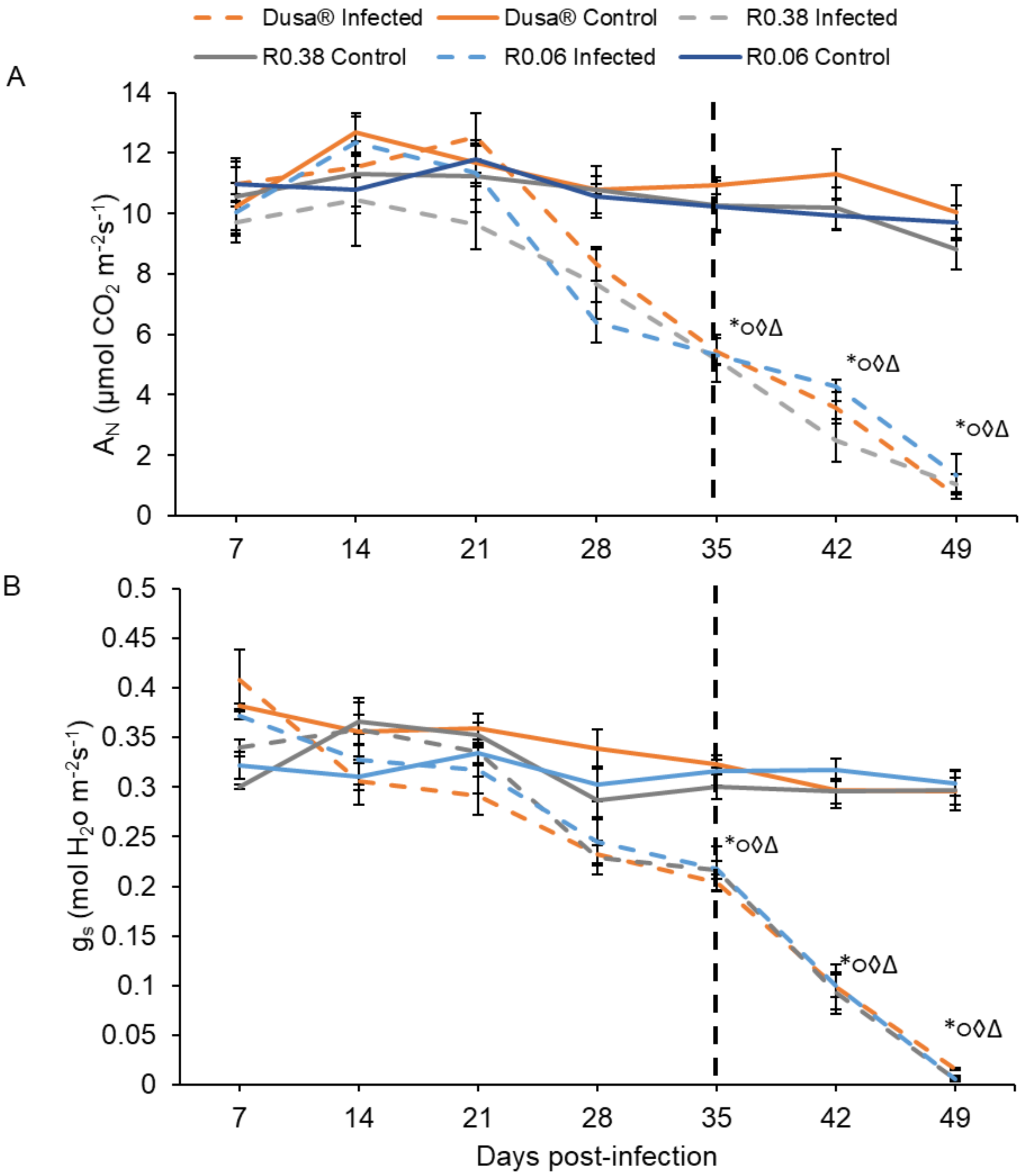

Figure 2.5: The impact of *Rosellinia necatrix* infection on net CO_2 assimilation (A_N) and stomatal conductance (g_s) of avocado rootstocks. Dotted lines represent infected plants, and solid lines show control plants of all the rootstocks. The black vertical dotted line separates the pre- and post-symptomatic stages. Asterisks indicate significance with symbols to denote the treatment/s for which differences were significant; 'Dusa®' (circle), R0.38 (diamond) and R0.06 (triangle). Bars indicate standard errors of the mean of 10 replicates and means were separated according to Tukey's LSD test (P<0.05).

The effect of R. necatrix *on intrinsic water use efficiency (iWUE) and the ratio of intercellular* CO_2 *to air* CO_2 *concentrations* (C_i/C_a) *of avocado plants*

There were no significant differences between infected and control plants of all the rootstocks before visible wilt symptoms of the leaves were apparent. A significant decrease in iWUE (Figure 2.6A) and C_i/C_a ratio (Figure 2.6B) of infected plants was evident at 42 dpi when compared to control plants of all three rootstocks. The reduction in iWUE and C_i/C_a ratio was accompanied by severe wilting of leaves of infected plants. All the rootstocks responded similarly and the decline of iWUE and C_i/C_a ratio of infected plants was continuous until the measurements ended at 49 dpi.

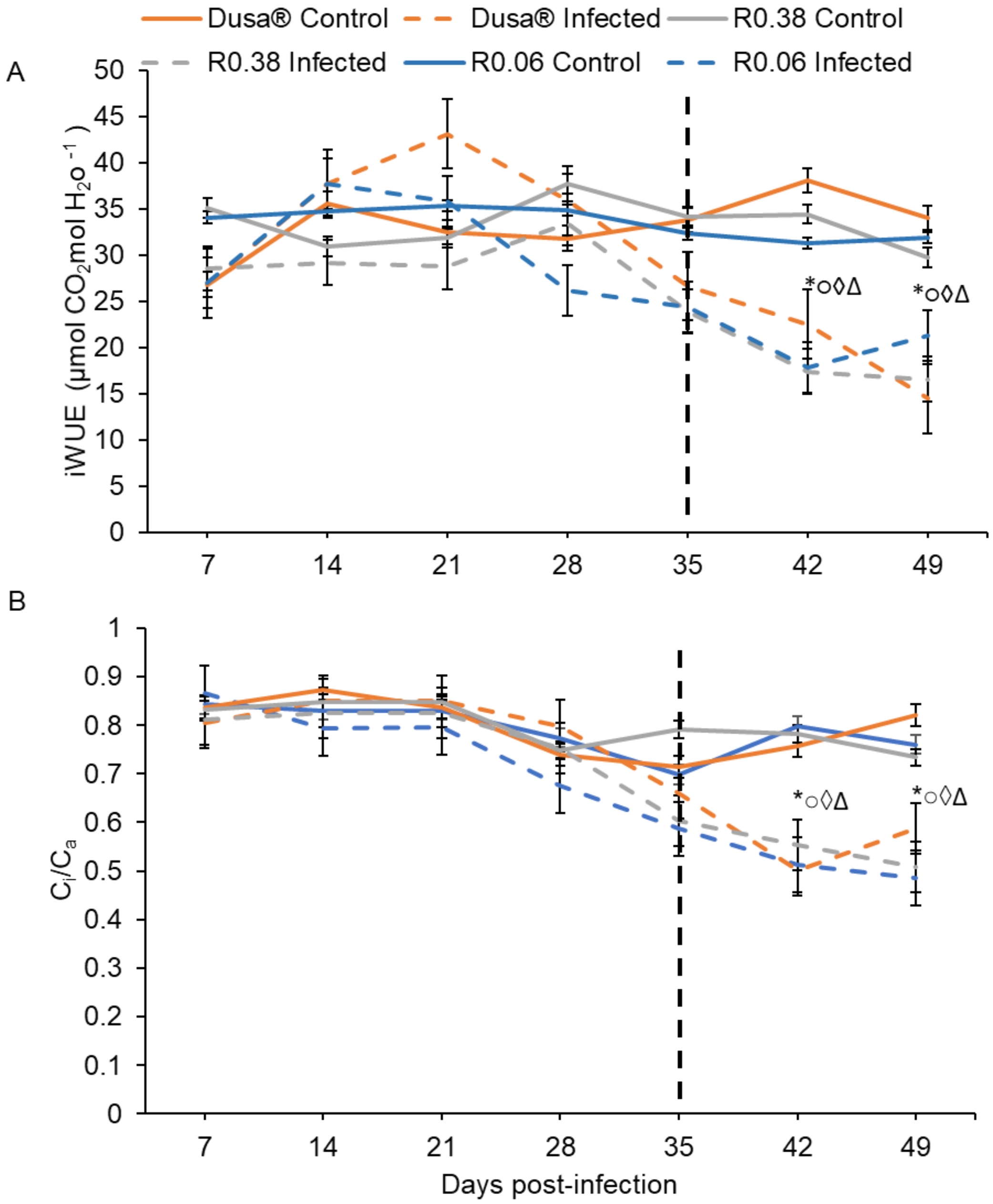

Figure 2.6: The impact of *Rosellinia necatrix* infection on intrinsic water use efficiency (iWUE) and the ratio of intercellular CO_2 to air CO_2 concentrations (C_i/C_a) of avocado rootstocks. Dotted lines represent infected plants, and solid lines show control plants in all the rootstocks. The black vertical dotted line separates the pre- and post-symptomatic stages. Asterisks indicate significance with symbols to denote the treatment/s which differences are significant; 'Dusa®' (circle), R0.38 (diamond) and R0.06 (triangle). Bars indicate standard errors of the mean of five replicates and means were separated according to Tukey's LSD test (P<0.05).

Assessment of chlorophyll fluorescence parameters in avocado rootstocks infected with *R. necatrix*

The effects of R. necatrix *on the maximum quantum efficiency of PSII (F_v/F_m ratio) and the efficiency of photosystem II (Φ_{PSII}) in avocado rootstocks*

The F_v/F_m ratio of infected plants declined significantly relative to control plants at 28 dpi, which was prior to the development of any visual stress symptoms as wilting of the leaves (Figure 2.7A). As WRR progressed, a further decrease in F_v/F_m ratio of infected plants was observed when the first wilt symptoms were evident at 35 dpi for all the rootstocks. The F_v/F_m ratio for healthy plants ranged between 0.75 and 0.83 throughout the trial, indicating that the plants were healthy. The F_v/F_m ratio of infected plants was below 0.70 from 28 dpi until the measurements were terminated.

From 35 dpi, Φ_{PSII} of infected plants showed significantly lower values than control plants when first visible stress symptoms were evident as wilting of the leaves (Figure 2.7B). As WRR progressed, the decline of F_v/F_m ratio and Φ_{PSII} was continuous following appearance of visible wilt symptoms of the leaves for infected plants and all the rootstocks responded similarly throughout the trial until the measurements ended at 42 dpi.

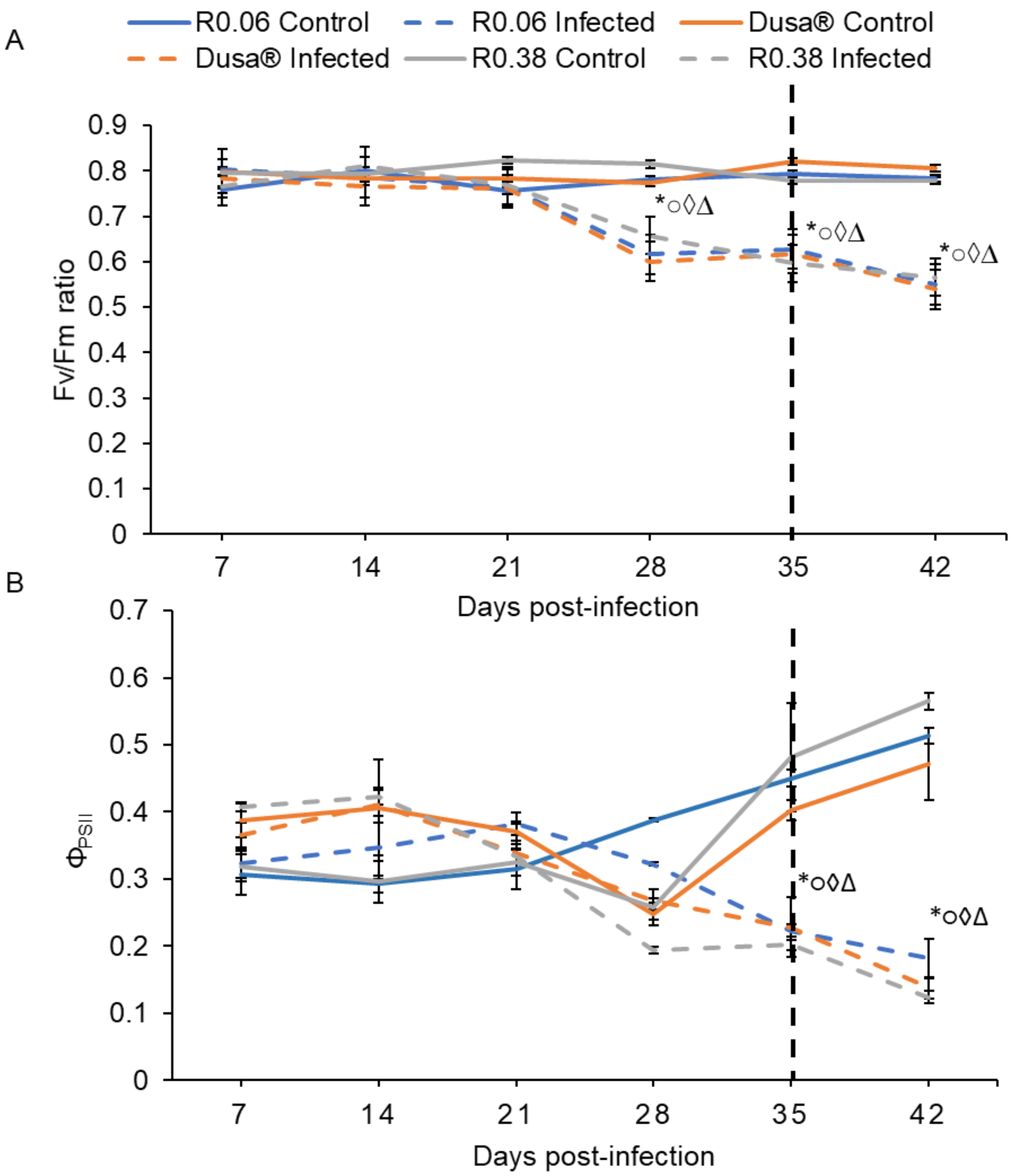

Figure 2.7: The impact of *Rosellinia necatrix* infection on the maximum quantum efficiency (F_v/F_m ratio) and the efficiency of photosystem II (Φ_{PSII}) of avocado rootstocks. Dotted lines represent infected plants, and solid lines show control plants in all the rootstocks. The black vertical dotted line separates the pre- and post-symptomatic stages. Asterisks indicate significance with symbols to denote the treatment/s which differences are significant; ‘Dusa®’ (circle), R0.38 (diamond) and R0.06 (triangle). Bars indicate standard errors of the mean of 10 replicates and means were separated according to Tukey’s LSD test ($P<0.05$).

The effects of R. necatrix *on photochemical (qP) and non-photochemical quenching (NPQ) in avocado plants*

The first significant increase in NQP (Figure 2.8A) and a decrease in qP (Figure 2.8B) of infected plants started at 28 dpi before visible wilt symptoms were evident for R0.06, however, also R0.38 demonstrated a significant decrease in qP compared to their controls. At this stage of infection, ‘Dusa®’ demonstrated no significant difference between infected and control plants. When first visible stress symptoms appeared at 35 dpi as wilt of the leaves; all the rootstocks of infected plants showed a significant increase of NPQ and a decreased of qP compared to their controls. At post-symptomatic stage, all the rootstocks responded similarly and NPQ continued to increase while qP decreased until the measurements ended at 42 dpi, when most of the infected plants had wilted as a result of *R. necatrix* infection.

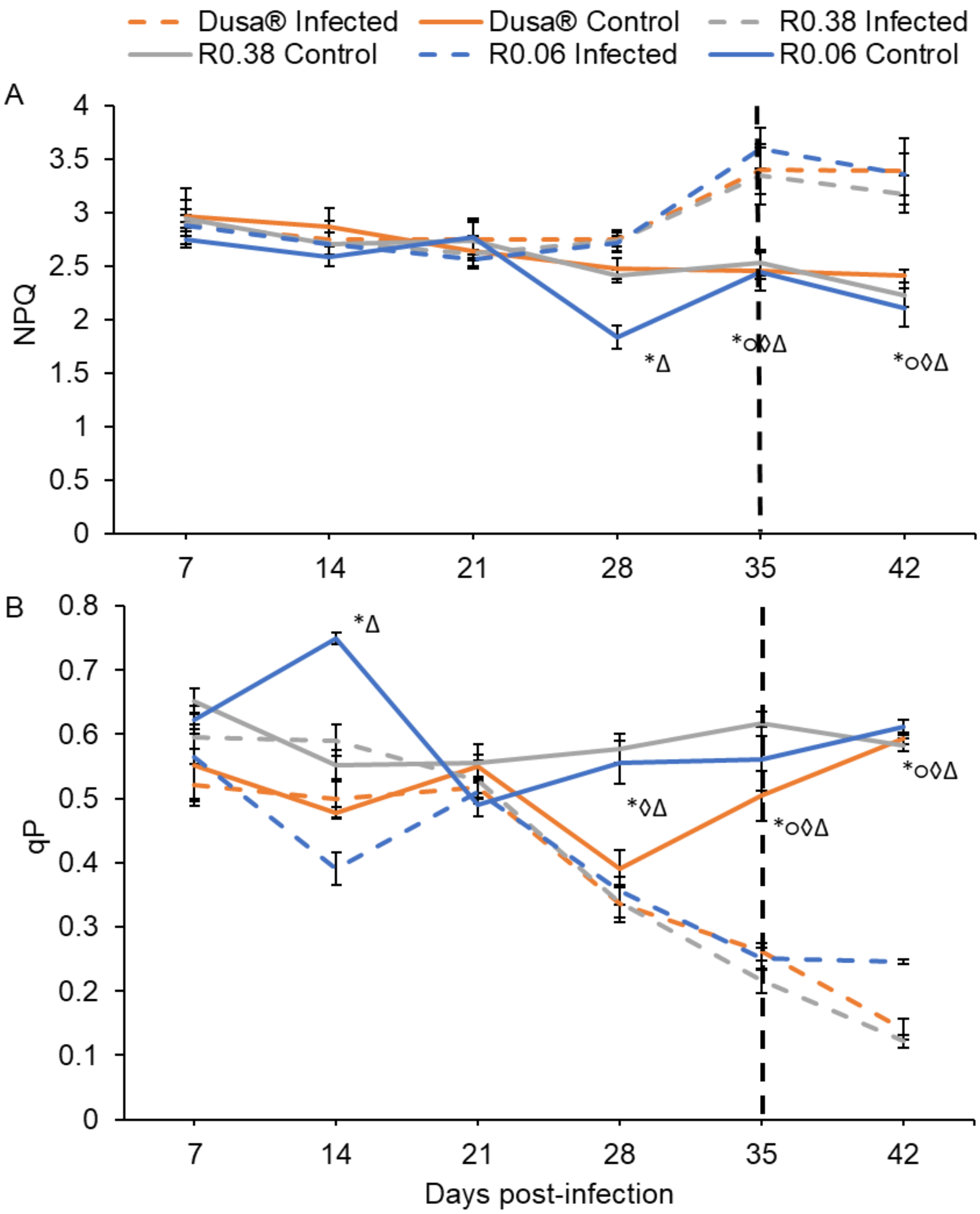

Figure 2.8: The impact of *Rosellinia necatrix* infection on non-photochemical quenching (NPQ) and photochemical quenching (qP) of avocado rootstocks. Dotted lines represent infected plants, and solid lines show control plants of all the rootstocks. The black vertical dotted line separates the pre- and post-symptomatic stage. Asterisks indicate significance with symbols to denote the treatment/s which differences are significant; 'Dusa®' (circle), R0.38 (diamond) and R0.06 (triangle). Bars indicate standard errors of the mean of 10 replicates and means were separated according to Tukey's LSD test (P<0.05).

Assessment of A-C$_i$ response curves in avocado plants infected with R. necatrix

The first changes in the initial slope and plateau in the A-C$_i$ response curves appeared at the pre-symptomatic stage for infected plants of all the rootstocks (Figure 2.9A). The A$_N$ showed a typical asymptomatic response to increasing C$_i$ in infected plants of all the rootstocks. The C$_i$ of infected plants failed to increase beyond 1400 µmol mol^1, while C$_i$ values of control plants continued to increase, approaching 1700 µmol mol^1. This difference was, however, not significant. At the post-symptomatic stage, there were significant differences between infected and control plants of all the rootstocks (Figure 2.9B).

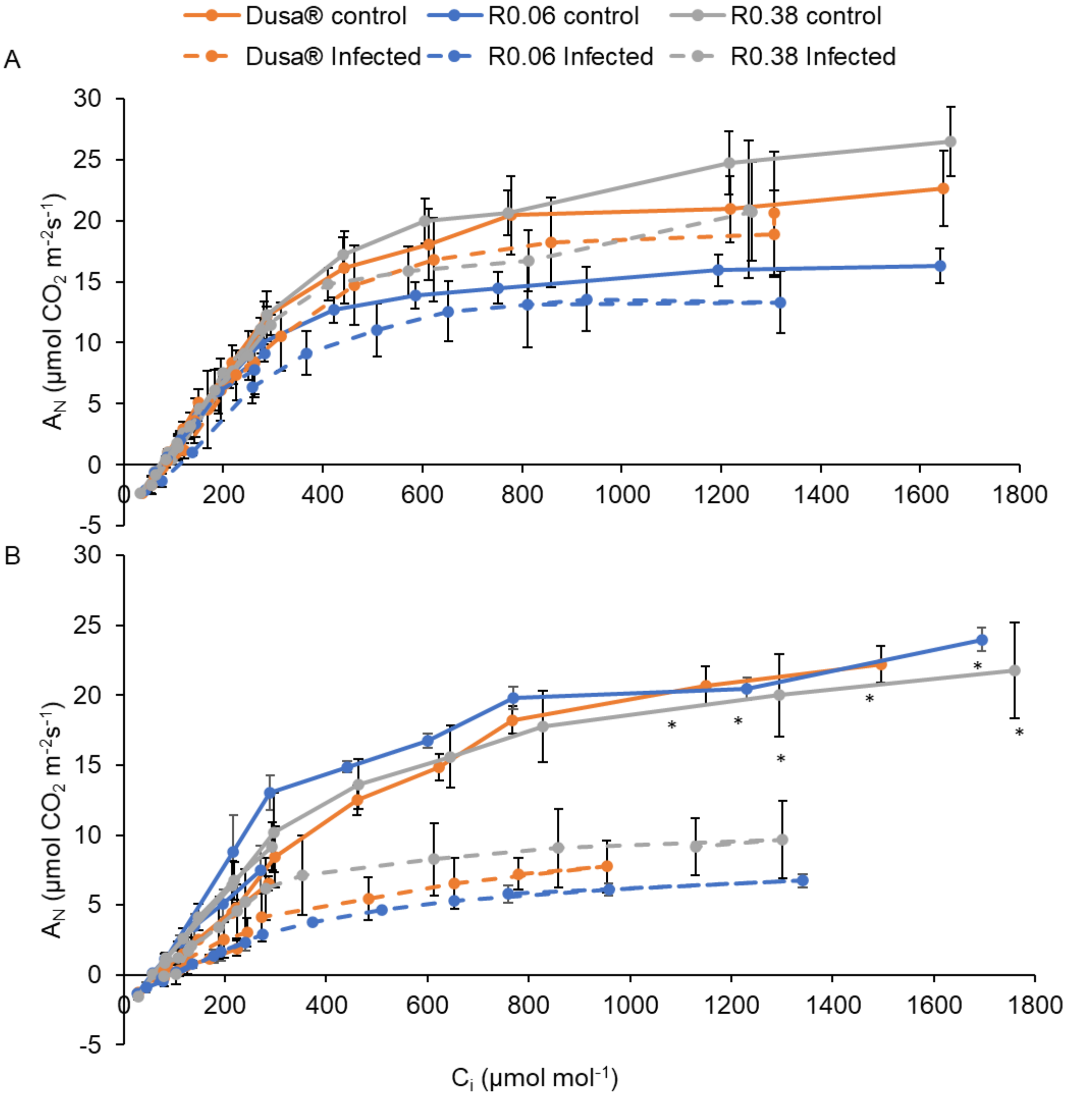

Figure 2.9: The impact of *Rosellinia necatrix* infection on the A-C$_i$ response curves of avocado rootstocks. Control (solid lines) and infected (dotted lines) avocado plants of the rootstocks at the pre-symptomatic stage (A) and post-

symptomatic stage (B). Asterisks indicate significance between infected and control plants of all the rootstocks. Bars indicate standard errors of the mean of five replicates and means were separated according to Tukey's LSD test (P<0.05).

The V_{cmax} and J_{max} of infected plants showed a significant difference compared to control plants when wilting symptoms of the leaves were evident at the post-symptomatic stage (Table 2.4). As WRR disease progressed, V_{cmax} and J_{max} limitations resulted in decreasing of both A_N and C_i of infected plants when compared to control plants (Table 2.4). Both V_{cmax} and J_{max} showed similar trends as the WRR disease progressed, all the rootstocks responded similarly between infected and control plants.

Table 2. 4: The effect of *Rosellinia necatrix* on the maximum rate of ribulose-1,5-bisphosphate carboxylation (V_{cmax}) and electron transport regeneration of ribulose-1,5-bisphosphate (J_{max}) in plants.

Stage of infection	Parameters	'Dusa®'		R0.38		R0.06	
		Control	Infected	Control	Infected	Control	Infected
Pre-symptomatic	V_{cmax}	42.22±30^{a}	37.27±20^{a}	49.50±11^{a}	45.16±12^{a}	43.68±7^{a}	35.09±18^{a}
	J_{max}	97.04±30^{a}	93.00±29^{a}	109.15±28^{a}	93.54±23^{a}	78.16±17^{b}	68.21±30^{b}
Post-symptomatic	V_{cmax}	32.71±5^{b}	16.93±2^{c}	47.82±4^{a}	17.03±1^{c}	48.74±9^{a}	13.51±0^{c}
	J_{max}	61.71±2^{b}	47.69±7^{c}	75.73±9^{a}	47.84±3^{c}	53.99±10^{b}	31.33±3^{d}

The letters represent significant differences between treatments of control and infected plants at pre- and post-symptomatic stage in all the rootstocks (P<0.05) and error bars represent the mean value of five replicates (±SE).

Discussion

Physiological responses of avocado rootstocks to *R. necatrix* have recently been investigated to provide insights to plant stress at the photosynthetic level. However, previous studies investigated leaf gas exchange and chlorophyll fluorescence parameters individually in different trials (Martínez-Ferri et al. 2016, Zumaquero et al. 2019b). In this regard, the current study used both parameters to assess the physiological response of three different avocado rootstocks to *R. necatrix* in the same trial. The purpose of the study was to determine if physiological changes occur at the pre-symptomatic stage which could facilitate early detection of stress in avocado rootstocks infected with *R. necatrix*. Currently there are no registered and fully effective control measures of *R. necatrix*, therefore early detection and rapid response are imperative to limit infection and spread to non-infected areas.

The detection of aerial WRR symptoms in infected plants of the three rootstocks ('Dusa®', R0.06 and R0.38) was first noted at 35 dpi as leaf wilting and most infected plants were dead in less than 60 days, when the trial ended. This is slightly later than a previous study which reported the first stress symptoms as wilting of the leaves at 28 dpi (Martínez-Ferri et al. 2016). The wilting of the leaves of infected plants was due to stress caused by the inability of the roots to transport water, as a result of mycelial growth in the vascular cells of the plants as reported in other studies (Pasini et al. 2016). In the current study, as WRR progressed on the roots, the development of white mycelia was associated with fungal colonization of the roots and the root collar, which likely obstructed water movement to above-ground parts of the plants. The root symptoms of WRR included the presence of white mycelia, with roots becoming necrotic and exhibiting root rot at later stages of infection of all the infected plants. A previous study had shown the same symptoms on the roots in susceptible avocado plants at the end of the trial confirming the effect of the pathogen (Martínez-Ferri et al. 2016). The presence of the pathogen in the current study was confirmed with re-isolation and PCR.

When WRR progressed, stomata were closed in the leaves of infected plants, which limited g_s and A_N, ultimately resulting in a decline in dry mass of infected plants. Stomatal closure is the first mechanism of plant defence during stress and an important factor controlling carbon fixation (Da Silva et al. 2008). The response of stressed plants to close stomata is likely to occur as a result of reduced water uptake as the pathogen affects the root structure and permeability of infected plants relative to control plants (Jokhan et al. 1996, Pezeshki 2001, Nicolás et al. 2005). Reduced growth and dry mass production suggest that *R. necatrix* caused primary limitations of infected plants. The lower values of dry mass of the roots when compared to the shoots may be caused by the fungus which killed the roots. This resulted in a very low R:S ratio of infected plants compared to control plants. Normally, plants under stress conditions often decrease their growth rate and dry mass production, which contributes more dry mass to the roots, to maintain a higher R:S ratio for regeneration of new roots (Yin et al. 2005). In the current study, the death of the roots due to *R. necatrix* contributed to the decline in R:S ratio of infected plants.

The photosynthetic rates indicate plant health and the capacity of the plant to survive under biotic and abiotic stress conditions. The present study indicated that *R. necatrix* stress led to a lower A_N value, which was similar to a previous study by Zumaquero et al. (2019b). This response was mainly due to stomatal closure, as manifested by reduced g_s. In this regard, root pathogens reduce water uptake in plants, resulting in stomatal closure to maintain leaf water status (Lawlor 2002). This causes a limitation to CO_2 diffusion into the leaf and results in a decline in A_N (Clemenz et al. 2008). A previous study reported an early and significant decrease in A_N and g_s in plants of 'Dusa®' rootstock after 22 dpi, before the development of aerial symptoms when infected with *R. necatrix* (Zumaquero et al. 2019b). Although a significant decrease in A_N and g_s was demonstrated in plants of 'Dusa®', R0.38 and R0.06 rootstocks at 35 dpi of infected plants in this study, following the appearance of the first visible stress symptoms as wilting of the leaves.

In contrast, *Rosellinia*-tolerant BG83 rootstock demonstrated no significant variation in gas exchange parameters as WRR progressed in a previous study (Zumaquero et al. 2019b). This showed the ability of the plants to counteract the effect of the pathogen on water relations, avoiding a decrease in A_N. The tolerance of BG83 to *R. necatrix* is most likely due to the ability to induce protease inhibitors and their negative regulators, including genes related to tolerance of salt and osmotic stress (Zumaquero et al.

2019b). In this study, the susceptibility of ‘Dusa®’, R0.06 and R0.38 is hypothesized to be linked to redox processes and cell-wall degradation activities, following rapid WRR progression decreasing the photosynthetic ability of the infected plants and, such behaviour has previously been reported in ‘Dusa®’ plants (Zumaquero et al. 2019b).

The current study further showed that a decrease in A_N and g_s was associated with a decrease in iWUE of infected plants relative to control plants because of changes in water relations. The reduction in iWUE in plants of ‘Dusa®’ relative to BG83 plants following infection with *R. necatrix* has been reported previously (Zumaquero et al. 2019b). The iWUE is an excellent indicator of plant growth and health under water limitation conditions (Liu and Stützel 2004), which is supported by reduced carbon production and stomatal closure in infected plants of the present study.

Stomatal closure limited CO_2 diffusion into the leaf of infected plants which resulted in a parallel decrease in C_i (Farquhar and Sharkey 1982). The C_i/C_a ratio of infected plants decreased at post-symptomatic stage relative to control plants. This suggests that the primary limitation of reduction in A_N of infected plants was due to stomatal limitation. This agrees with a previous study where ‘Dusa®’ plants infected with *P. cinnamomi* and exposed to flooding demonstrated a decrease in C_i/C_a ratio (Reeksting et al. 2014). In contrast, an increase in the C_i/C_a ratio in other studies indicated mesophyll capacity was associated with changes in chlorophyll content, changes in water and nutrient uptake, differences in enzyme efficiencies and damage to photosystems (Pezeshki 2001, Else et al. 2008, Reeksting et al. 2014).

Fluorescence measurements were used to further assess the photosynthetic performance of infected plants relative to control plants. In the present study, a significant decrease in the F_v/F_m ratio was observed at the pre-symptomatic stage of infected plants in all the rootstocks before visible wilting symptoms of the leaves were apparent. The F_v/F_m ratio is an indicator of plant stress (Maxwell and Johnson 2000) or photo-inhibition (Dias and Marenco 2006). The decline in F_v/F_m ratio of infected plants when exposed to pathogen stress, is likely to be caused by photo-inhibition. As consequences of stomatal closure, CO_2 in leaves of infected plants at early stages of infection was limited and resulted in changes of F_v/F_m ratio prior to development of wilting of the leaves. The changes in F_v/F_m ratio at post-symptomatic stage were supported by a significant decline in Φ_{PSII} of infected plants. The rapid progression of WRR following the first appearance of wilt symptoms in infected plants showed a significant effect on Φ_{PSII}, which caused a reduction in A_N. This reduction of Φ_{PSII} in infected plants of all the three rootstocks showed a decrease in the proportion of photosynthetic active radiation absorbed by chlorophyll associated with PSII, important for photochemistry (Maxwell and Johnson 2000).

The reduction of Φ_{PSII} was associated with a decrease in qP and increase in NPQ of infected plants which suggest an increase in protection through xanthophyll cycling. The process of xanthophyll cycling is an enzymatic cycle that plays a key role in stimulating energy dissipation within light-harvesting antenna proteins by NPQ - a mechanism to reduce the amount of energy that reaches the photosynthetic reaction centers (Müller et al. 2001). The decrease in qP of infected plants relative to control plants indicated a reduction of the proportion of reaction centers that are opened for photochemistry and a change in photochemical efficiency due to light saturation (Maxwell and Johnson 2000). This parameter indicates the onset of photo-inhibition and determines the level of photo-protective quenching of fluorescence (Baker and Oxborough 2004, Adams Iii et al. 2008, Baker 2008). In a healthy plant, values of NPQ range from 0.5 to 3.5 at saturating light intensities (Björkman and Demmig 1987, Maxwell and Johnson 2000). Control plants in the current study were within this range, but NPQ of infected plants was greater than 3.5 when above-ground wilt symptoms of the leaves were apparent.

The decrease in C_i values as WRR progressed due to stomatal closure resulted in changes in the A-C_i response curves due to decrease in A_N of infected plants, which gave rise to photo-inhibition due to lack of CO_2 fixation under saturation light. This damaged PSII of infected plants and resulted in a decline in V_{cmax} and J_{max}, which suggests non-stomatal limitations to photosynthesis at this stage. Non-stomatal limitations to A_N under-stress conditions in plants are associated with a reduction in Rubisco (Jia and Gray 2003) and RuBP regeneration (Tezara et al. 1999), and inhibition in the activity of PSII. This suggests that the decrease in V_{cmax} of infected plants could be as a result of reduced amounts of Rubisco (Nakano et al. 1997, Tissue et al. 1999), and at times to a low activation state (Sage 1994). The mechanism of lowering in J_{max} is less clear because the capacity of RuBP regeneration is determined through many biochemical steps in electron transport (Von Caemmerer and Farquhar 1981, Sudo et al. 2003) and the Calvin cycle (Sudo et al. 2003). Rubisco and RuBP regeneration limit the photosynthetic rate of plants at low and high CO_2 concentrations in stress conditions (Onoda et al. 2004).

Limitations of the current study was the large-scale trial under greenhouse conditions which made it difficult to take measurements using the open-path photosynthesis machine (LI6400XT), which requires more time and human resources. Due to time intervals between measurements, it was not possible to determine the exact time point when g_s and A_N started to decline, as measurements were taken once a week rather than daily. This affected the accuracy of stress detection before the visible WRR symptoms were evident.

Conclusion

South Africa is an important producer of avocados in the world, both for local consumption and export to European countries. To date, PRR has been the most serious biotic factor limiting production, with the sustainable production relying on using rootstocks partially resistant to *P. cinnamomi.* However, the presence of *R. necatrix* in avocado orchards is threatening the *P. cinnamomi*-partially resistant rootstocks. In this study, it is evident that 'Dusa®' a commercial rootstock was susceptible to *R. necatrix* infection, which was also comparable to both R0.38 and R0.06 avocado rootstocks. Infected plants exhibited stomatal closure in response to infection, which was manifested as a decrease in $g_{s,}$ which resulted in a decline in A_N relative to the controls. The significant reduction in A_N occurred concurrently with the appearance of visible leaf wilting of infected plants at 35 dpi. *R. necatrix* caused an impact on water uptake which resulted in a decrease of A_N; therefore, a change in carbon production led to a reduction in dry mass of infected plants relative to control plants of all three rootstocks. A decrease in A_N and g_s was associated with a decrease of iWUE in infected plants which indicated that the plants were not healthy following *R. necatrix* infection.

Infected plants had significantly lower values of F_v/F_m ratio before wilt symptoms as a result of stress caused by the pathogen. Stomatal closure of infected plants limited CO_2 diffusion into the leaf, which resulted in a decline in C_i and subsequently, a decrease in C_i/C_a ratio. Increased NPQ of infected plants was possible because of xanthophyll cycling as a strategy to avoid photosystem damaged as less light was partitioned to photochemistry due to a decrease in qP and Φ_{PSII}. Although stomatal limitations were major contributors to reduce A_N in infected plants, however, non-stomatal limitation started to occur at the post-symptomatic stage. These findings correlate with lower values of C_i/C_a ratio in infected plants of all the rootstocks. While fluorescence and A-C_i derived parameters decrease of infected plants as non-stomatal factors limiting A_N, but these changes were due to a decline in C_i of the leaf following stomata closure when pathogen stress increased over time causing photo-inhibition.

Currently, the detection of *R. necatrix* in orchards relies on human assessment of visual symptoms, followed by PCR confirmation assays. This method requires time and is expensive in terms of human resources and laboratory assays. Delayed detection of the pathogen allows the spread of the pathogen to surrounding plants and as a result relying solely on PCR detection is not feasible for early detection in large orchards. Therefore, the detection of the onset of stress in plants will play an important role in the identification of stressed plants for further investigation in avocado orchards, which would prompt early control and protection programs. The use of remote sensing techniques is highly recommended as a suitable approach to study the progress of *R. necatrix* infection at field level because these methods have been shown to offer precision in terms of promptness, sensitivity, and easy-to-use tools. Based on the current study g_s and chlorophyll flouerescence parameters like F_v/F_m ratio can be applicable in remote sensing at field level to detect stress in plants. There is still more work that needs to be done at the field level to be able to detect the physiological effects at the onset of stress in plants and prompt further investigation using PCR confirmation tools in order to diagnose *R. necatrix* as early as possible.

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CHAPTER 3

Assessing potential control strategies of White Root Rot on avocado

Abstract

White Root Rot (WRR) caused by *Rosellinia necatrix* is a severe threat to avocado production in areas where the pathogen is prevalent. As with Phytophthora Root Rot (PPR), an integrated and multifaceted approach is required to control WRR in avocado orchards. To date, no fully effective control method and no chemical or biological agents against *R necatrix* in South Africa for avocado. In this regard, this study aimed to investigate the potential of chemical control products (fluazinam and chloropicrin), and biological control agents (BCAs) against *R. necatrix.* The efficacy of BCAs (B-Rus, Beta-Bak, Mity-Gro^{TM} and *Trichoderma*), fluazinam and chloropicrin were evaluated *in vitro* and *in vivo* against *R. necatrix*. The findings showed that *Trichoderma* and B-Rus, fluazinam, and chloropicrin inhibited *R. necatrix* grown *in vitro*. *Trichoderma* and B-Rus suppressed WRR symptoms when applied before infection with *R. necatrix* in a greenhouse trial. In contrast, Mity-Gro^{TM} and Beta-Bak failed to inhibit the pathogen *in vitro* and *in vivo* in plants despite application of the products prior to infection with the pathogen. Fluazinam suppressed WRR symptoms in plants when applied at early stages of infection while chloropicrin rendered the pathogen non-viable when used as a pre-plant treatment. Plants treated with *Trichoderma*, B-Rus and fluazinam sustained dry mass production and net CO_2 assimilation by maintaining the green leaf tissues of the plants and generation of new roots for water uptake in the presence of WRR. This study has important implications on the integrated management of WRR in avocado orchards. The potential control strategies can prevent the spread of the disease in avocado orchards in the absence of tolerant rootstocks.

Keywords: Beta-Bak, B-Rus, Mity-Gro^{TM}, *Trichoderma*, Chloropicrin, Fluazinam, Rootstocks and White Root Rot.

Introduction

The discovery of partially resistant or tolerant rootstocks to *Phytophthora cinnamomi* Rands has made significant contributions to the world's fast-growing demand of avocado; however, root pathogens still cause significant loses of the fruit crop. *P. cinnamomi* remains the most limiting pathogen while *Rosellinia necatrix* Berl. ex. Prill. is now also considered an important root pathogen in avocado growing areas. These pathogens cause severe losses due to rootstocks susceptibility, overuse of nitrogenous fertilizers, inappropriate irrigation systems and regimes, lack of appropriate crop rotation and challenges faced when developing resistant rootstocks (Letourneau and Van Bruggen 2006, Chakraborty and Newton 2011, Gava and Pinto 2016). The lack of tolerant rootstocks and effective control methods against *R. necatrix*, the causal agent of White Root Rot (WRR) in avocado is problematic for the South African avocado industry and other growers around the globe where the pathogen is present.

WRR control remains a challenge in the field since *R. necatrix* spores can survive in acidic soils, are resistant to drought conditions and various fungicides (Ten Hoopen and Krauss 2006, Arjona-López et al. 2020) and are capable of penetrating deep within the soil (Pliego et al. 2012, Martínez-Ferri et al. 2016). Avocado production areas affected by WRR use soil solarization (Guillaumin 1986, López-Herrera et al. 1999), biological agents (Ruano-Rosa et al. 2014b) and chemical control methods (López-Herrera and Zea-Bonilla 2007, Ruano-Rosa et al. 2017, Arjona-López et al. 2020). Still, these methods have proven ineffective in completely eradicating the pathogen.

Chemical control methods involve the use of fungicides and soil fumigants with different modes of action. Fluazinam is the most popular fungicide tested against *R. necatrix* (López-Herrera and Zea-Bonilla 2007). Fungicides have not always yielded proper results due to microbial degradation but was still preferred for the control of soil-borne pathogens due to their effectiveness (Čadková et al. 2013). The mode of action of fluazinam is inhibiting spore germination and development of infection structures (Sugimoto 2002). In a previous study, fluazinam showed higher efficacy than carbendazim and thiophanate methyl both *in vitro* and *in vivo* against *R. necatrix*

(López-Herrera and Zea-Bonilla 2007*)*. A field study has shown that fluazinam reduced WRR disease in Japanese pear (Nitta et al. 1998). Recently, the fungicide was applied around infected avocado trees using a soil injector reaching a depth of 0.3 m, and a significant difference in tree health was observed after the first application between treated and non-treated plants (Arjona-López et al. 2020). A significant reduction of inoculum load of *R. necatrix* was demonstrated in treated soil when compared to non-treated soil (Arjona-López et al. 2020).

Chloropicrin has been used in pre-plant soil fumigation as a fungicide (Mao et al. 2014, Dangi et al. 2015). It is a general biocide soil fumigant that controls pathogenic bacteria, fungi, nematodes, and insects present in the soil (Li et al. 2017). The release of the fumigant in the soil is highly dependent on soil moisture and temperature (Ajwa et al. 2010). Increasing soil moisture levels and decreasing temperature reduces volatility rates of chloropicrin (Enebak et al. 1990, Ajwa et al. 2010). The effects of chloropicrin against *R. necatrix* in avocado orchards has not been investigated to date.

Biological control strategies for combating plant diseases are recommended in agriculture, although they may not always be as effective as fungicide treatments (Ghorbanpour et al. 2018). Biological control agents (BCAs) previously tested against *R. necatrix* in avocado plants included *Trichoderma* (Rosa and Herrera 2009, Ruano-Rosa et al. 2010, Ruano-Rosa et al. 2014b) and rhizobacteria species. (Cazorla et al. 2006, Pliego et al. 2011, González-Sánchez et al. 2013). *Trichoderma* isolates selected from avocado roots for their antagonistic effect against *R. necatrix* both *in vitro* and *in vivo* showed inhibition of the pathogen (Ruano-Rosa et al. 2003, Ruano-Rosa et al. 2010). Most of the *Trichoderma* isolates from avocado roots were characterized and identified as *Trichoderma atroviride*, *Trichoderma virens*, *Trichoderma harzianum* and *Trichoderma cerinum* (Ruano-Rosa 2006, Ruano-Rosa et al. 2010). Also, rhizobacteria species tested against WRR in avocado plants include *Bacillus subtilis* CB115 and *Pseudomonas chlororaphis* PCL1606 which showed reduced disease progression under greenhouse conditions (González-Sánchez et al. 2013). Another greenhouse trial reported that bacterial strains of *P. chlororaphis* PCL1606 and *B. subtilis* PCL1608 reduced WRR disease severity in avocado plants (Ruano-Rosa et al. 2014b). These bacterial strains demonstrated the ability to control

WRR and delay the appearance of visible aerial symptoms (Pliego et al. 2007, Pliego et al. 2012).

The current study aimed to investigate the effect of biological agents and chemical control products against *R. necatrix.* The study tested three hypotheses: (1) Application of *Trichoderma* and *Bacillus* species in avocado plants before infection with *R. necatrix* could reduce disease symptoms, sustain photosynthesis rate, and increase dry matter production relative to infected control plants. (2) Infected plants would not show visible aerial WRR symptoms when fluazinam is applied after infection with the disease, and (3) Chloropicrin could kill *R. necatrix* in the soil when used as a pre-plant treatment.

Materials and methods

Plant material

Twelve-month-old clonal avocado rootstocks (R0.06), susceptible to *R. necatrix*, with no grafted scion obtained from Westfalia Technological Services (WTS), Tzaneen, South Africa were transplanted into 5 L plastic bags containing a soil-perlite mixture (mixed at 1:1 ratio) and placed in a greenhouse on the experimental farm at the University of Pretoria, Pretoria, South Africa (25° 47' 7.38" S 28° 30.44" E). For the preparation and maintenance of the plants refer to Chapter 2.

Greenhouse conditions

Weather variables were recorded by a Campbell Scientific automatic weather station situated in the open approximately 300 m from the greenhouse. The average daily maximum temperature during the trial period was 27.9°C, with an average daily minimum temperature of 14.4°C. Average incoming solar radiation during the trial period was 19.80 MJ $m^{-2}day^{-1}$ and ranged from 5.36 to 30.29 MJ $m^{-2}day^{-1}$. The photosynthetically active radiation (PAR) in the greenhouse throughout the trial was on average of 321.7µmol $m^{-2}s^{-1}$, with a maximum of 918.22 µmol $m^{-2}s^{-1}$.

R. necatrix strain and inoculation

A virulent strain of *R. necatrix* (ARP-2017-Rn2) (van den Berg et al. 2018) previously isolated from avocado in South Africa was obtained from the culture collection of the Avocado Research Programme (ARP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. For the preparation of the isolate and inoculation procedures, refer to Chapter 2.

Biological control agents (BCAs)

The DSC Labs cc, Pretoria, South Africa supplied the ARP with four BCAs for the trial:

B-Rus

B-Rus contained Plant Growth Promoting Rhizobacteria (PGPR), *Bacillus subtilis* HC8, isolated from a giant hogweed *Heracleaum sosnowskyi* Manden (Malfanova et al. 2012) near Saint-Petersburg, Russia.

Beta-Bak

Beta-Bak contains the strain *Bacillus thuringiensis* subsp. *galleriae* PS. This bacterium was isolated from the growth tips of the roots of grass plants in South Africa by Prof. P.L. Steyn (DSC Lab cc) and identified with 16S rRNA profiling.

Mity-Gro^{TM}

Mity-Gro^{TM} contains the strain *Brevibacillus laterosporus* BOD, isolated from apple tree rhizospheres in China (Ruiu 2013). Another strain was isolated from tropical seawater on the Papua New Guinea coast and recently isolated from Mangrove marsh soil in India (Ruiu 2013).

Trichoderma

Trichoderma contains *Trichoderma harzianum* T22 and is produced as a formulation (Trianum G, Koppert, Berkel en Rodenrijs, The Netherlands) (Vitti et al. 2015). The root colonization by *T. harzianum* T22 and consequent plant-microbe interactions is profound in terms of plant disease control, growth, and productivity (Vitti et al. 2016).

Chemical control products

Brave

Brave (Fluazinum 500 g/l sc), supplied by Disa BioTechonologies cc (South Africa), has been tested against *R. necatrix* on avocado in Spain (López-Herrera and Zea-Bonilla 2007, Ruano-Rosa et al. 2017, Arjona-López et al. 2020). It is a broad-spectrum fungicide that inhibits germination of fungal spores.

Chloropicrin (Pic Plus)

Trical Africa (South Africa) supplied the ARP with a soil fumigant, chloropicrin. The soil fumigant is important in agriculture against many soil pathogens, including *Rhizoctonia*, *Verticillium*, *Phytophthora* and *Pythium* species (Li et al. 2017).

Antagonistic effects of BCAs against *R. necatrix* grown *in vitro*

The antagonistic activity of BCAs; B-Rus, Beta-Bak, Mity-Gro^{TM} and *Trichoderma,* were evaluated against *R. necatrix*, respectively in dual culturing assays on 90-mm

Petri plates containing $\frac{1}{2}$ PDA. A 5-mm agar disc from a 5-day-old culture of the *Trichoderma* (Figure 3.1A), was placed opposite a 5-mm agar disc from a 7-day-old culture of *R. necatrix* (20 mm away from the plate periphery). For the remaining bacterial products (Figure 3.1B), a 5-mm agar disc from a 7-day-old culture of *R. necatrix* was placed at the centre of each Petri plate. Single bacterial colonies from 5-day-old cultures were placed in four different positions, 20 mm away from the periphery of the plate. Each bacterial product was evaluated on a separate plate. Control plates included *R. necatrix* only. Culture plates were incubated at 25°C in the dark for 8 days, and radial growth of *R. necatrix* was measured every second day. Percentage inhibition (PI) of mycelial growth was calculated using Equation 3.1 (Vincent 1947);

$$\text{Percentage inhibition} = 100\text{x}\frac{\text{C} - \text{T}}{\text{C}} \qquad (3.1)$$

Where PI= percentage of inhibition; C= diameter (cm) of radial growth of *R. necatrix* in control and T= diameter (cm) of radial growth of *R. necatrix* in the presence of antagonistic strains.

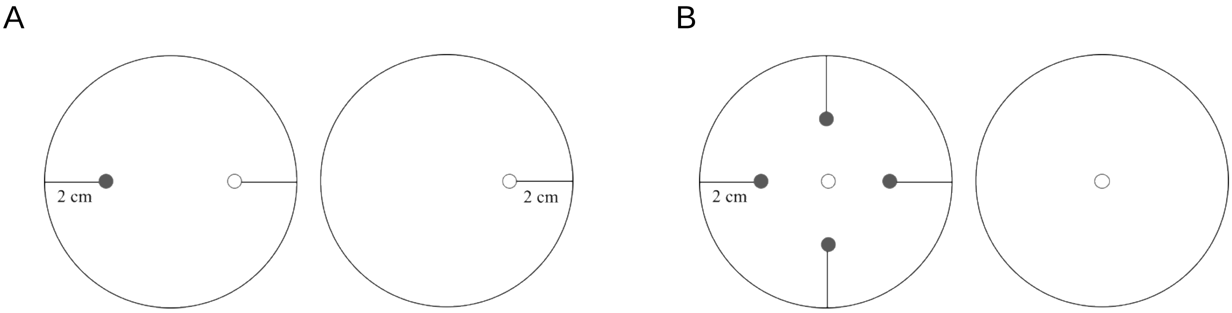

Figure 3.1: *In vitro* dual culturing of *Rosellinia necatrix* and candidate biological control agents. (A) A 5-mm disc of *Trichoderma* was placed opposite a 5-mm disc of *R. necatrix*, 20 mm away from the periphery of the plate, a second plate contained only *R. necatrix*. (B) A 5-mm disc of *R. necatrix* was placed in the centre of the plate, with four single bacterial colonies inoculated 20 mm away from the periphery of the

plate, while the second plate only contained *R. necatrix*. The BCAs are represented by the black circles and *R. necatrix* by the clear circles**.**

Efficacy of fluazinam and chloropicrin against *R. necatrix* grown *in vitro*

Fluazinam was supplemented into cooled $\frac{1}{2}$ PDA at the following concentrations: 0.05, 0.1 and 0.15 µg/mL, respectively; before being poured into 90-mm Petri plates. A 5-mm agar disc from a 7-day-old *R. necatrix* culture was inoculated onto the fluazinam supplemented plates and incubated at 25°C in the dark for 8 days. Ten replicate plates for each concentration of fluazinam were prepared. Colony growth was measured every second day for 8 days to assess inhibition of the pathogen. Percentage inhibition of mycelial growth was calculated as previously described using Equation 3.1.

The efficacy of chloropicrin against *R. necatrix* grown on $\frac{1}{2}$ PDA was assessed by inoculating plates with a 5-mm agar disc of *R. necatrix* mycelia and incubated at 25°C in the dark for 7 days. In a fume hood, 1 mL/L of chloropicrin was pipetted into the petri plates which were opened 45º for the treatment of *R. necatrix* culture that were growing in the plates. The *R. necatrix* cultures used for controls were pipetted with distilled water. Following the treatment, plates were incubated in the dark for 3 days at 25°C. Then 5-mm discs of *R. necatrix* mycelia were cut from the treated plates and transferred to fresh $\frac{1}{2}$ PDA and incubated at 25°C in the dark for 7 days. Mycelial growth was measured every 2 days. Ten replicates were assessed for both the treatment and control, respectively.

The efficacy of chloropicrin against *R. necatrix* grown inside wood pieces was also assessed. A hole was drilled into the centre of 8 wood pieces (20 mm by 5 mm), before soaking in distilled water for 24 h and autoclaving. Sterile needles were used to inoculate *R. necatrix* mycelia into the wood pieces, which were then sealed in 500 mL containers (2 wood pieces per container) and incubated at ambient room temperature in the dark for approximately 21 days to allow the pathogen to colonize the wood. The different chloropicrin concentrations (0.25, 0.5 and 1 mL/L, respectively) were pipetted into 20 mL bottles. These bottles were then placed inside three separate containers (1 L) with *R. necatrix* inoculated pieces of wood. For the control, a 20 mL bottle containing 1 mL distilled water was placed in the fourth container. The containers were resealed and placed in the fume hood at ambient room temperature for 14 days. The wood pieces were then cut open, and four fragments were sampled per treatment. These samples were surface sterilized using 70% ethanol for 30 s and washed with distilled water before placing them on sterile Petri plates containing about 20 mL of $\frac{1}{2}$ PDA. The samples were incubated at 25°C in the dark upright and inverted after 24 h for 7 days.

Assessment of BCAs and fluazinam on *R. necatrix*-infected plants

BCAs were applied 14 days prior to inoculating the soil of avocado plants with *R. necatrix* and every 14 subsequent days following inoculation until termination of the trial (Table 3.1). For a subset of plants, B-Rus was only applied 14 days after inoculation to test its capabilities as a post-infection treatment. BCA solutions were prepared by adding 15 g of each formulation in 10 L of water, respectively. A solution of fluazinam was prepared by adding 0.1 µg/mL of fluazinam into 1 L of water. Fluazinam was applied 14 days after infection and every subsequent 14 days until the trial was terminated. The application rate for fluazinam and BCAs was 50 mL solution per plant. A total of 10 avocado plants were included per treatment (Table 3.1).

Table 3.1: The descriptions and abbreviations of the products tested for control of *Rosellinia necatrix.*

Treatment	Description	Application
Mity-Gro^{TM} (MG)	*Brevibacillus laterosporus*	Pre- and post-infection
Beta-Bak (BB)	*Bacillus thuringiensis*	Pre- and post-infection
B-Rus (BR)	*Bacillus subtilis*	Pre- and post-infection
Trichoderma (Tricho)	*Trichoderma harzianum* isolate T22	Pre- and post-infection
B-Rus (BRpost)	*Bacillus subtilis*	Post-infection only
FLU	Fluazinam	Post-infection only
Rn	*Rosellinia necatrix*	Non-treated plants
Chloropicrin	Soil fumigant	Pre- infection

Assessment of WRR disease development in avocado treated with BCAs and fluazinam

The health of 10 avocado plants per treatment was assessed by monitoring aerial disease development weekly and assessing the roots at 84 days of the trial period. The scores of both aerial and root symptoms and calculation were done as described in Chapter 2.

Assessment of biomass (dry mass) in *R. necatrix*-infected plants treated with BCAs and fluazinam

The dry mass of roots, stems and leaves was determined for each plant following oven drying at 70°C for 3 days. When the trial ended, the number of green leaves attached to the plant were counted, and the stem length was measured. Calculation of root: shoot ratio (R:S) and the relative water content (RWC), was done as in Chapter 2.

Detection of *R. necatrix* in the roots using re-isolation and PCR confirmation

Root material from infected control plants, uninfected control plants and all the treatments were harvested at 84 days when the trial was terminated and frozen for use in PCR assays. For the preparation and isolation of fresh root samples for culturing, visualization of cultures under light microscope and extraction of DNA from all the samples, refer to Chapter 2.

Assessment of leaf gas exchange in *R. necatrix*-infected plants treated with BCAs and fluazinam

The plants were assessed for photosynthetic performance throughout the trial by measuring leaf gas exchange [net CO_2 assimilation (A_N) and stomatal conductance (g_s)] in avocado plants, The measurements were recorded weekly on five replicates per treatment from mid-morning until afternoon (10:00 - 14:00 h) on the first fully expanded mature leaves from the top of each plant. Measurements of leaf gas exchange parameters were conducted as described in Chapter 2.

Assessment of chloropicrin for the control of *R. necatrix* in plants

An independent trial was conducted using 40 plants of 12-month-old clonal avocado rootstock (R0.06) with no grafted scion from WTS as previously described in chapter 2 of this dissertation. *R. necatrix* inoculum was prepared as described in Chapter 2. Buckets containing 5 kg of sandy soil were watered and infected with *R. necatrix* (20 pieces of colonized chopsticks in the soil of each bucket). After soil infection, the buckets were kept in the greenhouse for a month, by which time white mycelial growth appeared on the soil surface before applying chloropicrin. A concentration of 1 mL/L of chloropicrin was used into 5 kg of soil. The buckets were sealed for 7 days and then opened to allow for volatilization and degradation of the soil fumigant. A subset of buckets considered for the controls were treated with distilled water rather than the soil fumigant. Avocado plants were transferred to 5 L plastic bags containing the treated and untreated soil from the buckets mixed with perlite (1:1 ratio). Soil samples were collected from both the chloropicrin treated and untreated soils to test for the presence of *R. necatrix* by baiting with avocado twigs and leaves. For the disease scoring of WRR symptoms and DNA extraction procedure for the soil samples (30 mg per sample) refer to Chapter 2.

Statistical analysis

All BCAs and chemical products were tested *in vitro* against *R. necatrix* using 10 replicates plates per treatment and average growth of mycelia was compared. For greenhouse trials 10 plants from each treatment were used to calculate the disease severity and dry mass partitioning. Five plants were randomly selected per treatment for leaf gas exchange analysis. The mean values of each variable for leaf gas exchange analysis were hence derived from only five plants. Microsoft Excel and statistical analysis (SAS) 9.4 version were used to identify significant differences between treatments. The results were subjected to analysis of variance (ANOVA) and the means of all treatments were separated using Tukey's test and the Least Significant Difference (LSD) both at a 5% level of significance ($p<0.05$).

Results

Antagonistic effects of BCAs against *R. necatrix* grown *in vitro*

The different bacterial products showed variable inhibition rates; BR inhibited the growth of the pathogen significantly, which failed to colonize the Petri plates after 8 days, at which time the plates containing only *R. necatrix* were overgrown (Figure 3.2A). BB and MG both failed to inhibit the pathogen, and *R. necatrix* completely colonized the Petri plates despite the presence of these BCAs (Figure 3.2A). No significant difference was observed between the *R. necatrix* only and either the MG and BB, with a growth rate of the pathogen above 80% (Figure 3.2B). However, BR was significantly different from all the treatments, with less than 20% growth rate of the pathogen (Figure 3.2B).

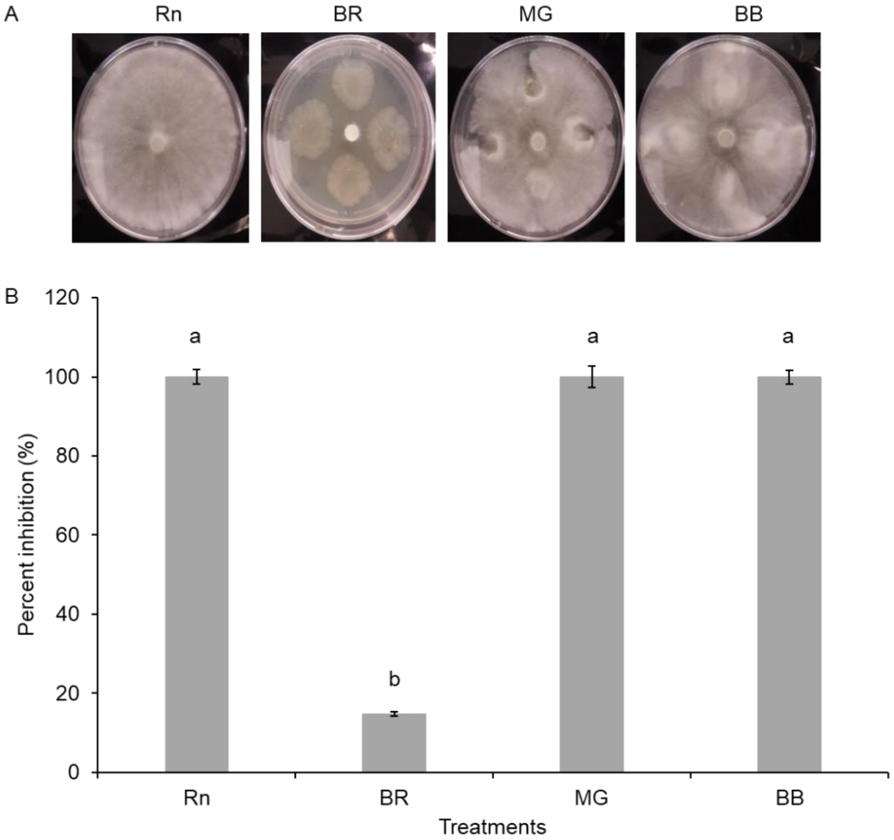

Figure 3.2: Antagonistic effects of different bacterial products against *Rosellinia necatrix* in a dual culture assays shown percent inhibition of mycelia at 8 days post-inoculation. (A) Inhibition of *R. necatrix* by different bacterial products in dual culture. *R. necatrix* only control (Rn), B-Rus (BR), Mity-Gro^{TM} (MG), and Beta-Bak

(BB). (B) The graph represents data collected at 8 dpi when the *R. necatrix* only control (Rn) completely colonized the plates. Bars indicate standard errors of the mean of 10 replicates and means with the same letter were not significantly different according to Tukey's LSD test (P<0.05).

Tricho was also evaluated for *in vitro* antagonism against *R. necatrix* using a dual culture technique. Tricho inhibited the growth of the pathogen and completely covered the plates within 5 days (Figure 3.3A). The product significantly reduced the growth rate of the pathogen to less than 20% (Figure 3.3B). The BCAs of Tricho and BR demonstrated the ability to inhibit the pathogen when compared with BB and MG when grown *in vitro*.

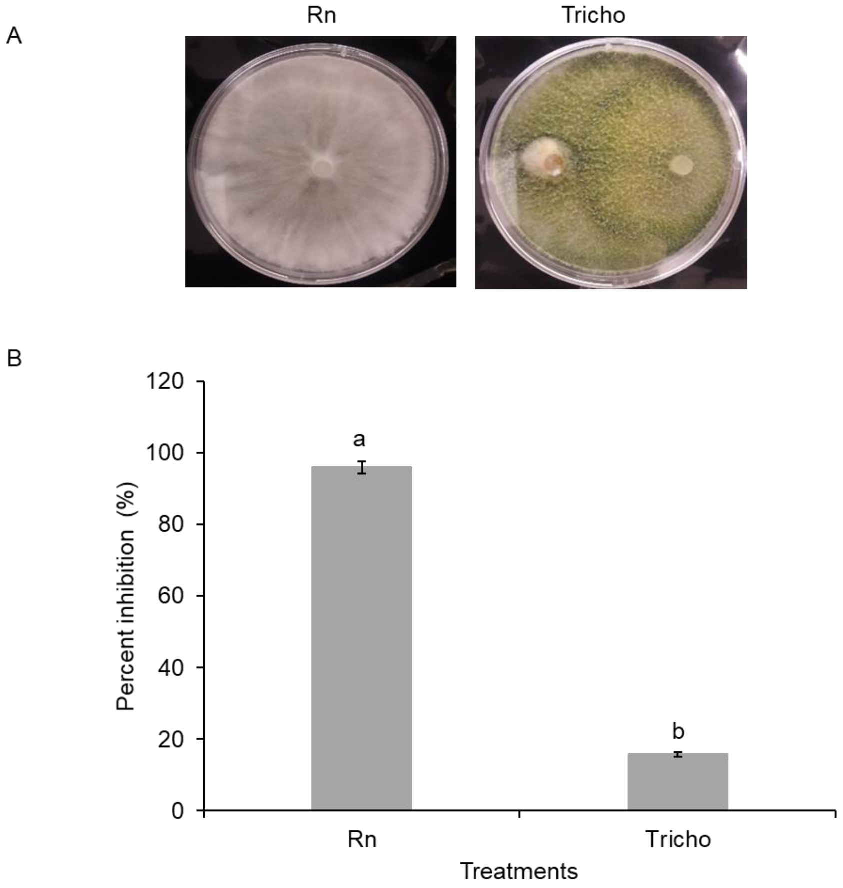

Figure 3.3: Antagonistic effects of *Trichoderma* against *Rosellinia necatrix* in a dual culture assay shown percent inhibition of mycelia at 8 days post-inoculation. (A) *R. necatrix* only control (Rn) and inhibition of *R. necatrix* by *Trichoderma* (Tricho) in dual culture. (B) The graph represents data collected at 8 days

when *R. necatrix* covered the plates*.* Bars indicate standard errors of the mean of 10 replicates and means with the same letter were not significantly different according to Tukey's LSD test (P<0.05).

Efficacy of fluazinam against *R. necatrix* grown *in vitro*

No mycelial growth was observed on plates treated with different concentrations of FLU in the first 3 days after inoculation. At 4 dpi, *R. necatrix* growth was observed on plates with the lowest FLU concentration, while growth on plates with 0.1 µg/mL and 0.15 µg/mL only appeared at 6 dpi (Figure 3.4A). The fungicide was found to be effective against *R. necatrix* at all three concentrations and demonstrated only 22, 19 and 17% growth, respectively (Figure 3.4B). When the trial ended at 8 dpi, all FLU concentrations had significantly reduced the growth of the pathogen in culture compared to untreated *R. necatrix* control plates.

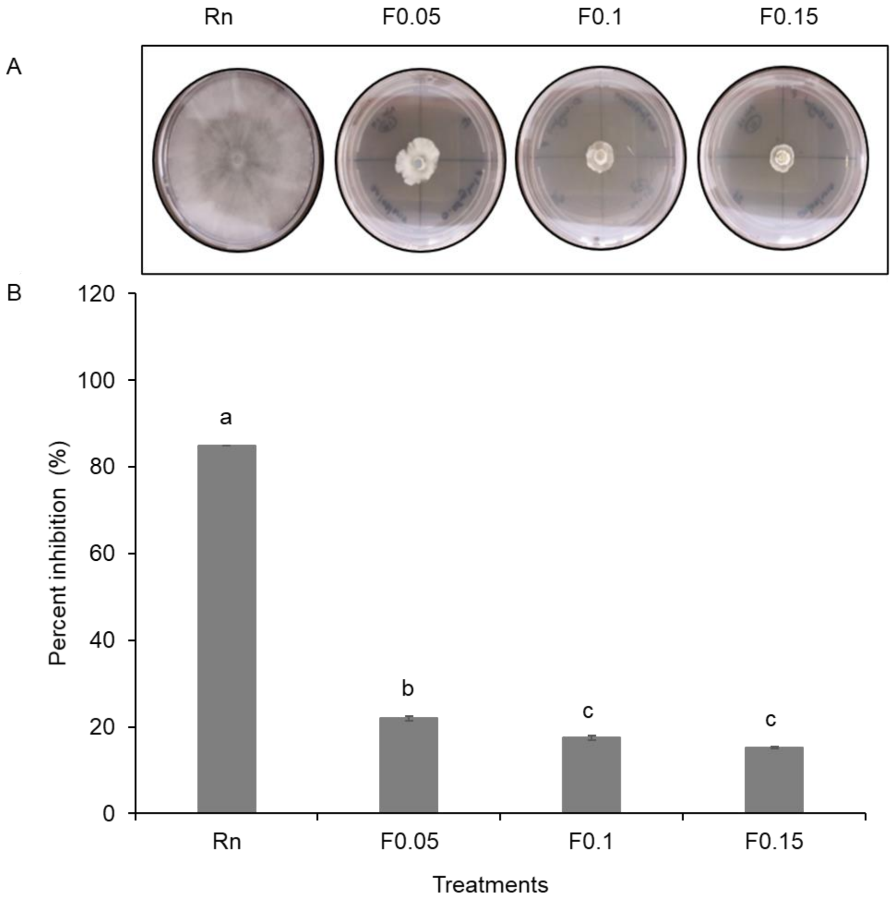

Figure 3.4: Efficacy of different concentrations of fluazinam against *Rosellinia necatrix* at 8 dpi. (A) *In vitro* percent inhibition of mycelia on $\frac{1}{2}$ PDA treated with fluazinam and untreated control. **(B)** The graph represents data collected at 8 dpi when

R. necatrix covered the plates. Bars indicate standard errors of the mean of 10 replicates and means with the same letter were not significantly different according to Tukey's LSD test (P<0.05). Rn = untreated *R. necatrix* control and treated with F0.05 = 0.05, F0.1 = 0.1, F0.15 = 0.15 µg/mL of fluazinam, respectively.

Efficacy of chloropicrin against *R. necatrix* grown *in vitro*

R. necatrix mycelia developed from plates of the untreated control (Figure 3.5A) while cultures treated with chloropicrin demonstrated non-viable mycelia (Figure 3.5B). None of the cultures transferred from the chloropicrin treated plates showed *R. necatrix* growth even at 22 dpi. The addition of 1 mL/L of chloropicrin killed the *R. necatrix* cultures.

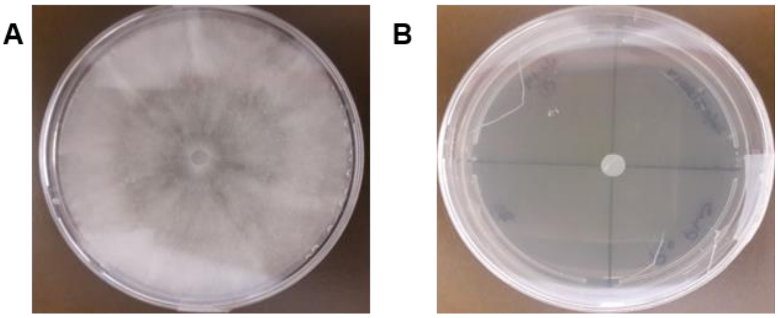

Figure 3.5: Efficacy of chloropicrin against *Rosellinia necatrix* when inoculated onto $\frac{1}{2}$ PDA. (A) *R. necatrix* colonized the control plates (distilled water) 8 dpi. (B) No growth of *R. necatrix* mycelia observed on the chloropicrin treated plates, even after 22 dpi. The results were consistent for all 10 replicated plates.

The ability of chloropicrin to penetrate woody material

R. necatrix white mycelia colonized the untreated wood pieces (Figure 3.6A), while no white mycelial growth was observed on the chloropicrin treated wood pieces (Figure 3.6B, C and D). Untreated wood fragments in culture plates demonstrated white mycelial growth (Figure 3.7A), but no growth was observed from the wood fragments treated with chloropicrin (Figure 3.7B, C and D). Even at the lowest doses of chloropicrin, the fumes penetrated the wood pieces and killed *R. necatrix*.

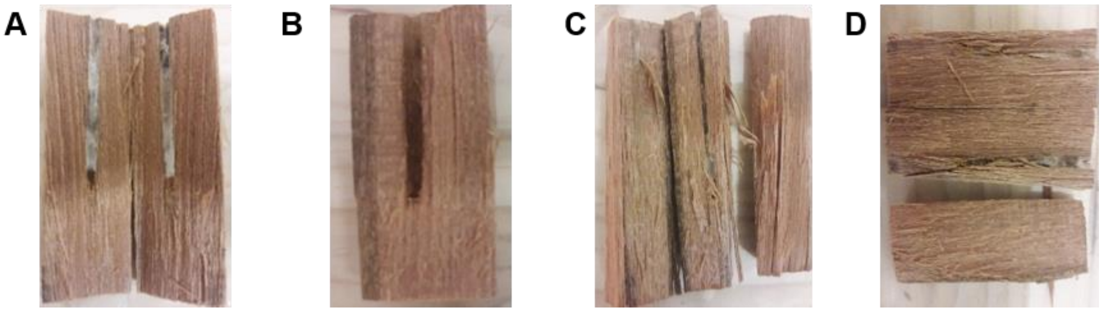

Figure 3.6: Penetration effects of chloropicrin inside wood material infected with *Rosellinia necatrix*. Wood pieces previously inoculated with *R. necatrix* and exposed to different volumes of chloropicrin. (A) untreated *R. necatrix* control (1 mL distilled water); (B) 0.25, (C) 0.5 and (D) 1 mL/L of chloropicrin, respectively. After 14 days of incubation, the wood pieces were cut in half. White mycelial growth was observed on the untreated *R. necatrix* control wood pieces (A), while all the treated wood pieces were clear of visible *R. necatrix* growth (B - D).

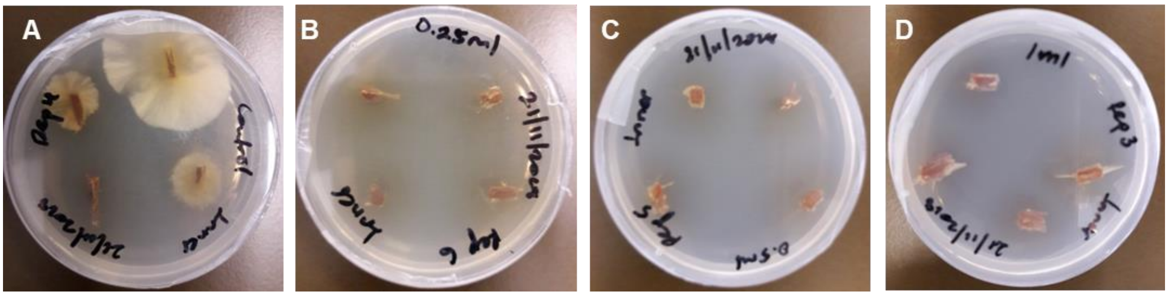

Figure 3.7: Growth of *Rosellinia necatrix* from wood pieces treated with chloropicrin and untreated *R. necatrix* control samples. Wood pieces from all the treatments were plated onto $\frac{1}{2}$ PDA and incubated for 7 days in the dark at 25°C. White mycelia developed from the untreated control wood samples (A). No mycelial growth originated from the pieces obtained from the treated wood pieces (B. 0.25, C. 0.5, and D. 1 mL/L of chloropicrin, respectively).

Assessment of BCAs and fluazinam for the control of *R. necatrix*

Detection of R. necatrix *in the roots using re-isolation and PCR confirmation*

No fungal growth was observed on root material isolated from uninfected control plants (Figure 3.8A). *R. necatrix* was successfully re-isolated from diseased avocado roots of infected control plants (Figure 3.8B) and from all the treatments (Figure 3.8C - G). Viable *R. necatrix* was purified successfully from all the treatments and infected control plants (Figure 3.8H). PCR results confirmed the identity of *R. necatrix*. The expected band size was approximately 500 bp using species-specific primers for *R. necatrix* (Figure 3.9). The symptoms observed in the plants were therefore due to *R. necatrix* infection*.*

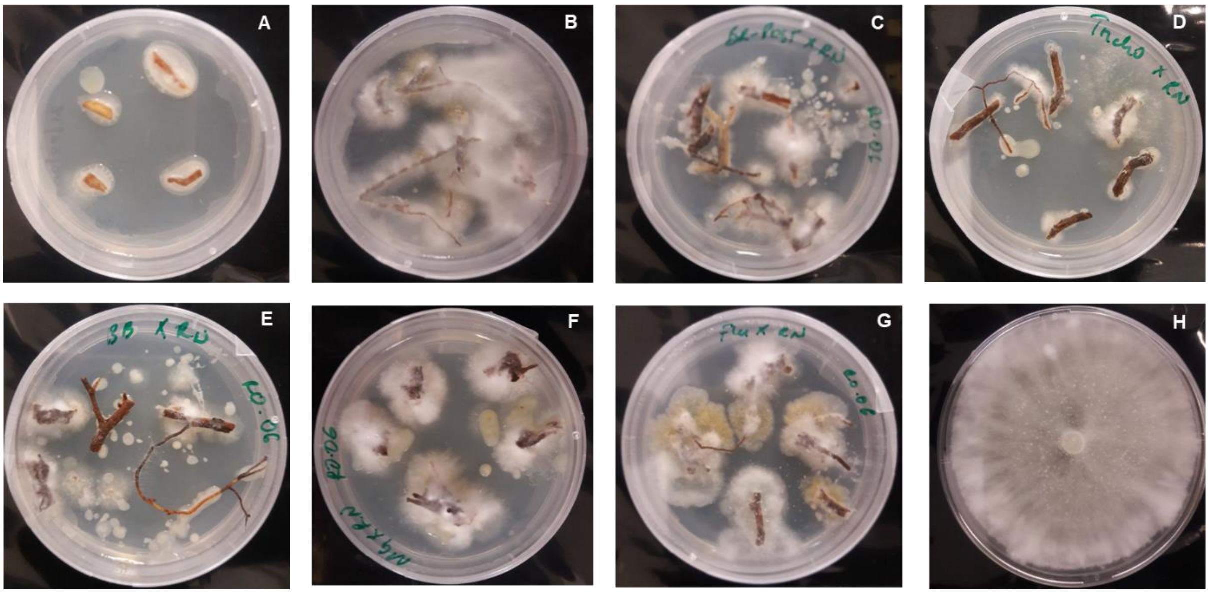

Figure 3.8: The mycelial growth of *Rosellinia necatrix* was plated out on $\frac{1}{2}$ PDA from infected plant root material treated with biological control agents and fluazinam. (A) Uninfected control plant roots and no fungal growth was observed. (B) Infected control plant roots. Roots isolated from Infected plants and treated with; (C) B-Rus (BR), (D) *Trichoderma* (Tricho), (E) Beta-Bak (BB), (F) Mity-Gro^{TM} (MM) and (G) fluazinam (FLU). (H) Purified culture of *R. necatrix* grown from the infected root materials.

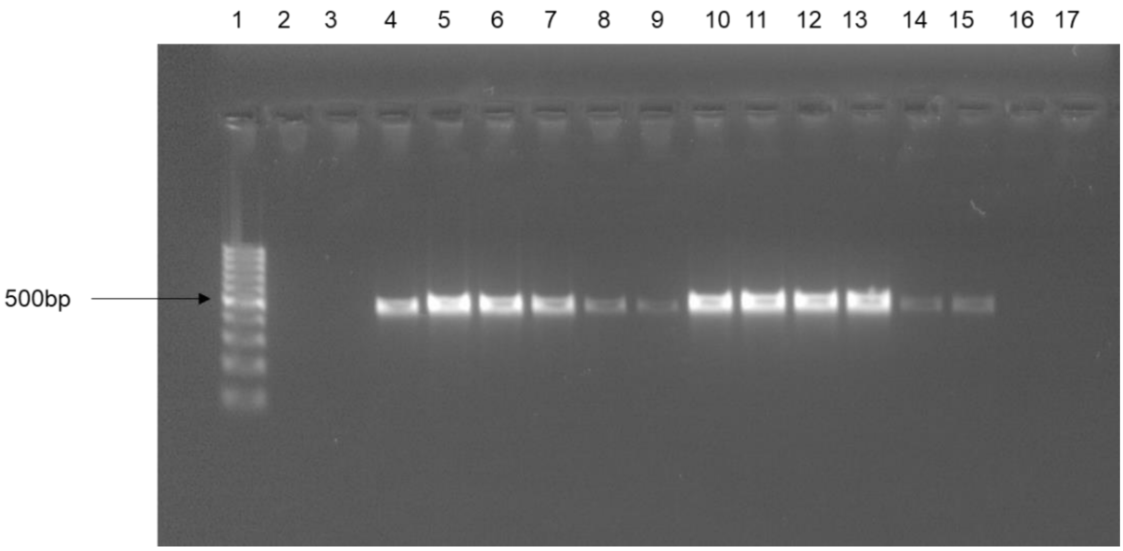

Figure 3.9: PCR confirmation assays of *Rosellinia necatrix* isolated from avocado from all the treatments. The R3 and R8 *R. necatrix* species-specific primers confirmed the presence of the pathogen in root samples of the plants treated with biological control agents and fluazinam. The presence of a ~500 bp band confirms the presence of *R. necatrix* DNA. 1. 100 bp molecular marker; 2 and 3. Uninfected control samples; 4 and 5. infected control samples; 6 and 7. B-Rus treated samples; 8 and 9. *Trichoderma* treated samples; 10 and 11. Beta-Bak treated samples; 12 and 13. Mity-Gro^{TM} treated samples; 14 and 15. Fluazinam treated samples and 16 and 17. Non-template control.

Assessment of BCAs and fluazinam in R. necatrix*-infected plants*

Plants treated with MG, BB and infected controls showed the first symptoms of stress at 42 days after infection. At this stage of the experiment, no signs of stress were observed on uninfected control plants and plants treated with Tricho and BR. WRR progressed rapidly in the infected control plants and plants treated with Mity-Gro^{TM} and BB, which all showed wilted leaves at 56 days. Uninfected control plants did not demonstrate any signs of stress until the experiment ended at 84 days (Figure 3.10A (A)) while infected controls (Figure 3.10A (B)) demonstrated severe signs of stress as wilting and desiccation. On the contrary, plants treated with Tricho (Figure 3.10A (C)) and BR (Figure 3.10A (D)) remained healthy with green leaves present and only moderate leaf drop as a sign of stress at 84 days. At 84 days, treated plants of BB (Figure 3.10A (E)) and MG (Figure 3.10A (F)), demonstrated severe signs of stress as wilting and desiccation of the leaves. Plants treated with FLU (Figure 3.10A (G)) showed similar response to plants treated with Tricho and BR. However, plants treated with BR-post-treatment (Figure 3.10A (H)) demonstrated severe signs of stress as plants treated with BB and MG. Disease severity of plants treated with BB, MG and BR-post-treatment was between 70 to 73% compared to the 80% of infected controls (Figure 3.10B). Treated plants of Tricho, FLU and BR showed 48, 35 and 45% disease severity, respectively (Figure 3.10B), which was significantly lower when compared with the other treatments.

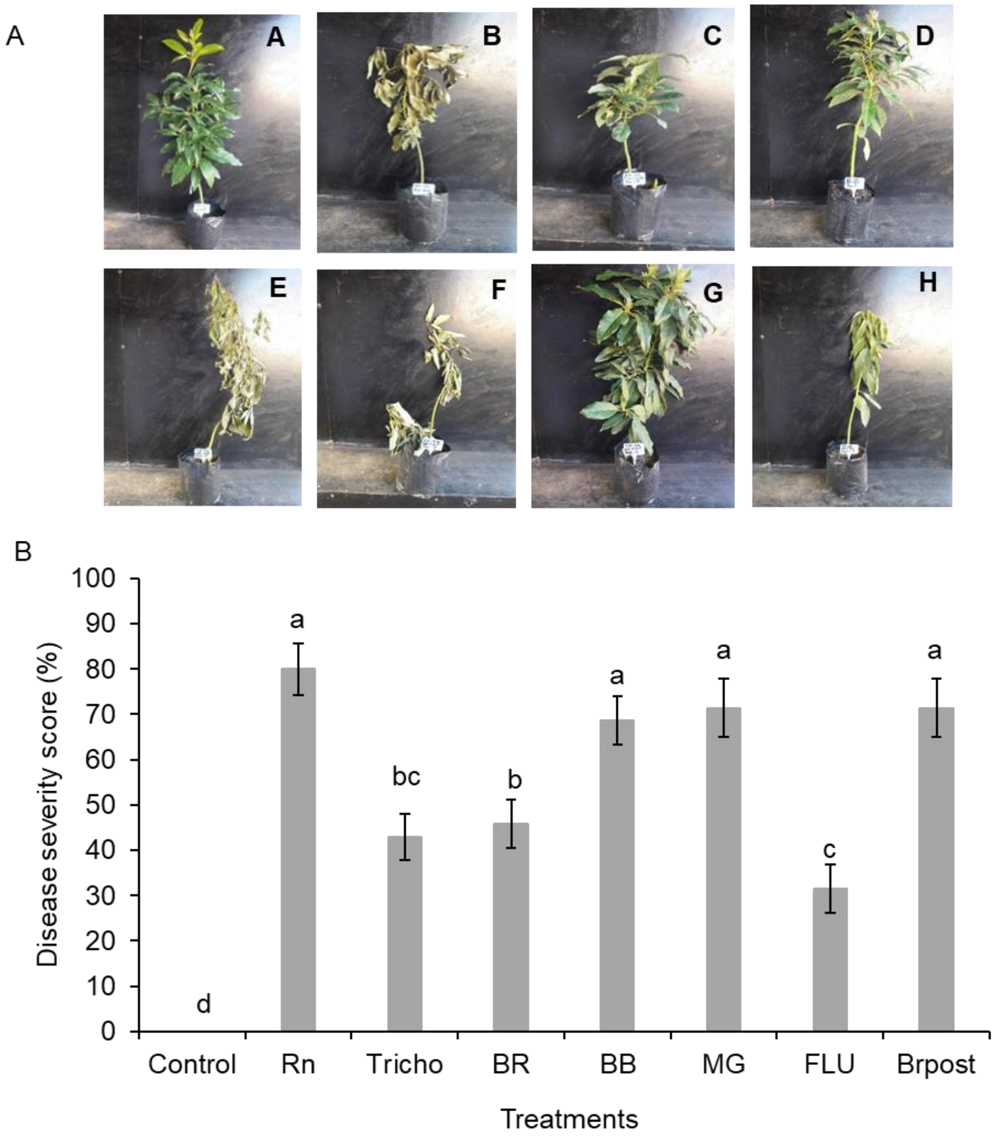

Figure 3.10: Visual assessment of aerial White Root Rot symptoms on plants treated with biological control agents and fluazinam recorded at 84 days. (A) WRR symptoms on plants exposed to eight different treatments; Uninfected control (A), infected control (Rn; B), *Trichoderma* (Tricho; C), B-Rus (BR; D), Beta-Bak (BB; E), Mity-Gro^{TM} (MG; F), fluazinam (FLU; G) and B-Rus post (BR-post; H). (B) The

graph shows the percentage disease severity of *R. necatrix* on above-ground plant parts for the different treatments. Bars indicate standard errors of the mean of five replicates and means with the same letter were not significantly different according to Tukey’s LSD test (P<0.05).

At 84 days of the experiment, uninfected control plants demonstrated no sign of root rot and necrosis (Figure 3.11A (A)). Infected control plants had black, brittle roots covered by white mycelia (Figure 3.11A (B)). Plants treated with Tricho (Figure 3.11A (C)) and BR (Figure 3.11A (D)) showed less rotten and necrotic roots. Plants treated with BB (Figure 3.11A (E)) and MG (Figure 3.11A (F)) showed root symptoms with severe necrotic tissues and rotten roots. While plants treated with FLU (Figure 3.11A (G)) showed similar response to plants treated with Tricho and BR while plants treated with BR-post-treatment (Figure 3.11A (H)) were severely affected by the disease. Disease severity assessment of the roots showed that uninfected control plants had 0% necrosis and were significantly different from all the treatments. Plants treated with BR, Tricho and FLU showed 55, 55 and 30%, respectively while plants treated with BB and MG showed 75 and 80% disease severity (Figure 3.11B). Infected control plants demonstrated 90% disease severity and treated plants of BR-post-treatment showed 62%.

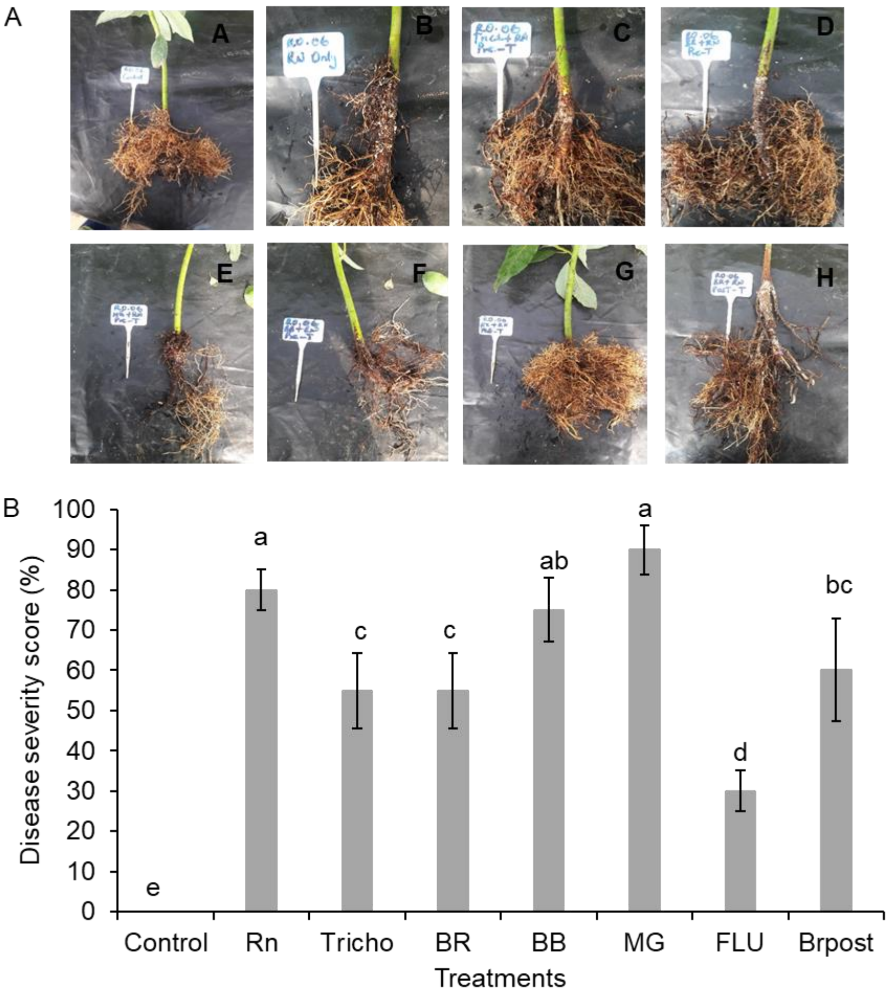

Figure 3.11: Visual assessment of White Root rot symptoms on the roots at 84 days. (A) WRR symptoms on plants exposed to eight different treatments; Uninfected control (A), infected control (Rn: B), *Trichoderma* (Tricho; C), B-Rus (BR; D), Beta-Bak (BB; E), Mity-Gro^{TM} (MG; F), fluazinam (FLU; G) and B-Rus-post (BR-post; H). (B) The graph shows the percentage disease severity of *R. necatrix* on root tissues. Bars indicate standard errors of the mean of five replicates and means with the same letter were not significantly different according to Tukey's LSD test (P<0.05).

Assessment of R. necatrix *effect on the biomass (dry mass) of the plants treated with BCAs and fluazinam*

Infected control plants showed a significant decrease in leaf, stem, and root dry mass relative to plants treated with Tricho, BR, FLU, and BR-post-treatment (Table 3.2). While plants treated with BB and MG had the lowest dry mass, which was significant when compared to the other treatments. Plants treated with Tricho, BR, FLU, and BR-post-treatment showed an increase in dry mass of the leaves, stems and roots when compared to that of uninfected control plants. Plants treated with BB, MG and infected controls demonstrated lower values of root dry mass as a result of severe necrosis and death of roots compared to the other treatments.

A significant difference was observed in R:S ratio and RWC of plants treated with Tricho, BR, FLU and BR-post-treatment compared to plants treated with MG, BB and infected control plants at the end of the trial. Uninfected control plants demonstrated the highest R:S ratio compared to all the treatments. The RWC showed that plants treated with Tricho, BR and FLU were not significantly different from uninfected controls (Table 3.2). Wilting and desiccation of the plants play an essential role in the reduction of dry mass production and allocation.

Table 3. 2: Assessment of *Rosellinia necatrix* effect on the dry mass of avocado treated with biological control agents and fluazinam. Means of stem, leaf and root tissue, root:shoot ratio (R:S) and relative water content (RWC) of treatments for Control – Uninfected control, Rn- Infected controls, Tricho - *Trichoderma*, BR- B-Rus, BB- Beta-Bak, MG - Mity-Gro^{TM}, BR-post - B-Rus-post and FLU - fluazinam.

Variable	Control	Rn	Tricho	BR	BB	MG	BR-post	FLU
Leaf (g)	24.9±2.5^{b}	18.5±1.2^{d}	32.7±2.5^{a}	34.5±3.5^{b}	24.6±2.0^{d}	22.9±2.5^{d}	31.6±10.8^{a}	30.7±3.2^{a}
Stem (g)	22.7±1.3^{a}	16.0±1.5^{d}	19.2±1.2^{abc}	21.3±3.5^{ab}	16.9±2.0^{d}	13.5±1.5^{e}	20.3±2.2^{abc}	19.6±2.7^{abc}
Root (g)	21.4±2.1^{a}	10.2±2.1^{d}	14.3±1.6^{c}	17.9±1.2^{ab}	10.5±1.6^{d}	4.7±1.9^{e}	19.5±1.3^{a}	14.9±1.6^{c}
R:S	0.4±0.0^{b}	0.2±0.0^{d}	0.4±0.0^{b}	0.5±0.3^{a}	0.2±0.0^{d}	0.1±0.0^{e}	0.3±0.0^{c}	0.3±0.0^{c}
RWC	7.6±1.0^{c}	4.7±0.9^{d}	9.3±0.7^{bc}	11.2±1.1^{bc}	4.4±1.2^{d}	2.0±1.3^{e}	5.2±0.9^{c}	7.1±1.1^{c}

Different letters indicate significant differences in the same row between control and infected plants in each rootstock based on a Tukey's LSD statistical test ($P<0.05$).

Assessment of leaf gas exchange measurements in *R. necatrix*-infected plants treated with BCAs and fluazinam

Leaf gas exchange results presented in this section considered plants treated with B-Rus, *Trichoderma,* and fluazinam. These treatments influenced changes in net carbon assimilation (A_N), stomatal conductance (g_S) and intrinsic water use efficiency (iWUE) when compared to infected and uninfected control plants.

The effect of R. necatrix *infection on net CO_2 assimilation (A_N) in plants*

Net CO_2 assimilation of infected control plants was significantly different to the treated plants and uninfected control plants after 56 days (Figure 3.12). This was shown by the appearance of visible symptoms such as wilting and subsequent abscission of the leaves of infected control plants. Following the development of symptoms in infected control plants, the disease progressed rapidly and A_N continued to decline until the measurements ended at 84 days. Uninfected controls were significantly different to the treatments at the end of the trial. All the treatments responded similarly when compared to the uninfected and infected control plants.

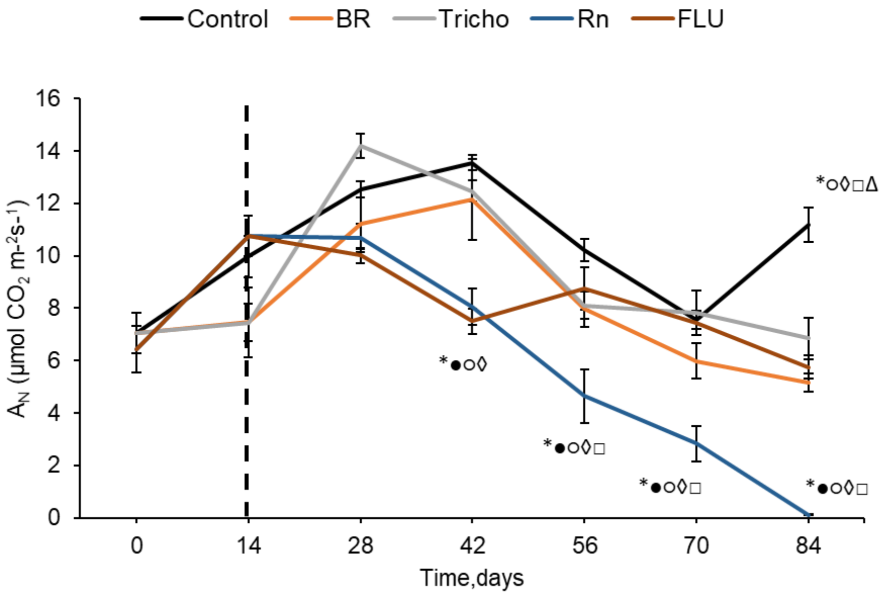

Figure 3.12: The effects of *Rosellinia necatrix* on net CO_2 assimilation in plants treated with B-Rus (BR), *Trichoderma* (Tricho) and fluazinam (FLU)*.* The dotted vertical line at 14 days represents the infection of the plants with *R. necatrix*. Asterisks indicate significance with symbols to denote the treatment/s where differences are significant; uninfected control (black circle), FLU (Square), Tricho (diamond), BR (clear circle) and infected control (Rn) (triangle). Bars indicate standard errors of the mean of five replicates and means were separated according to Tukey's LSD test (P<0.05).

The effect of R. necatrix *infection on stomatal conductance (g_S) in plants*

A significant difference of g_S was observed when infected control plants showed signs of stress as wilting of the leaves at 56 days compared to the treated plants and uninfected controls. (Figure 3.13). As WRR progressed, following the development of above-ground symptoms as wilting of the leaves, the decline of g_S was continuous in infected control plants compared to the treated and uninfected control plants until the end of the measurements at 84 days. The treatments and uninfected controls responded similarly, and no significant difference was observed until the measurements ended.

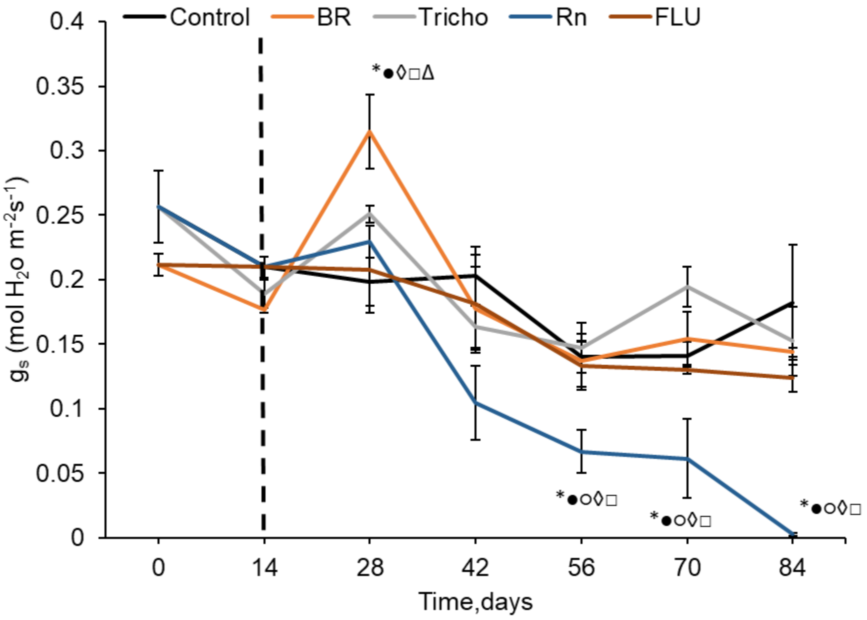

Figure 3.13: The effects of *Rosellinia necatrix* on stomatal conductance in plants treated with B-Rus (BR), *Trichoderma* (Tricho) and fluazinam (FLU). The dotted vertical line at 14 days represents the infection of the plants with *R. necatrix*. Asterisks indicate significance with symbols to denote the treatment/s where differences are significant; uninfected control (black circle), FLU (Square), Tricho (diamond), BR (clear circle) and infected control (Rn) (triangle). Bars indicate standard errors and means were separated according to Tukey's LSD test (P<0.05).

The effect of R. necatrix *infection on intrinsic water use efficiency (iWUE) in plants*

Infected control plants showed a significant difference in iWUE at 42 days compared to the treatments, except for plants treated with fluazinam (Figure 3.14). At 56 days, all the treatments were significantly different from infected control plants when the first sign of stress as wilting of the leaves was evident. Uninfected control plants did not demonstrate signs of stress throughout the trial, as shown by the g_s values. Infected plants showed severe symptoms as wilting at 70 days and continued to decline in iWUE until the measurements were terminated at 84 days. The treatments and uninfected controls responded similarly, and no significant difference was observed until the measurements ended.

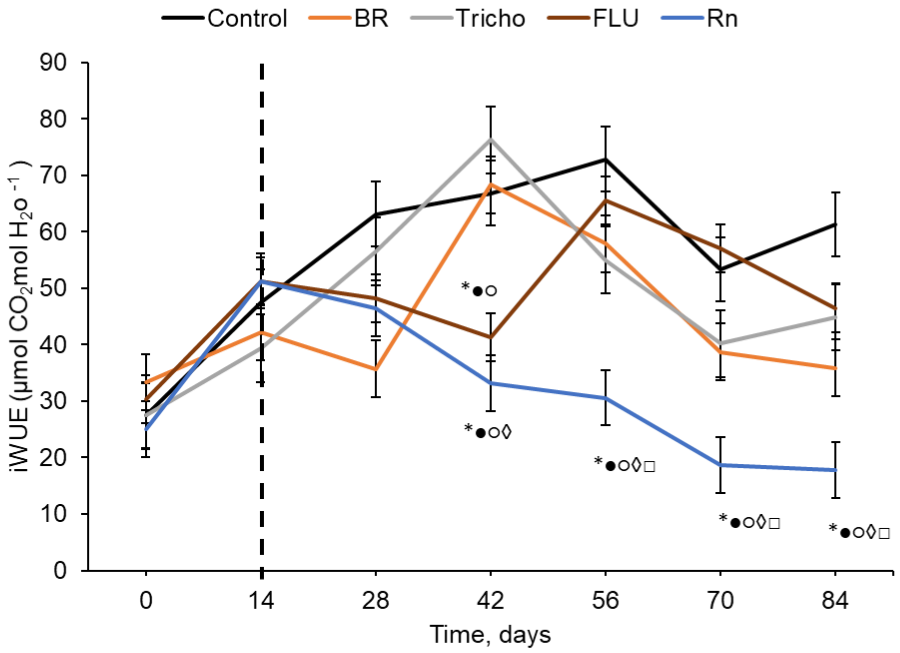

Figure 3.14: The effects of *Rosellinia necatrix* infection on intrinsic water use efficiency in plants. The dotted vertical line at 14 days represents the infection of the plants with the pathogen. Asterisks indicate significance with symbols to denote the treatment/s where differences are significant; uninfected control (black circle), FLU (Square), Tricho (diamond), BR (clear circle) and Infected control (Rn) (triangle). Bars indicate standard errors of the mean of five replicates and means were separated according to Tukey’s LSD test (P<0.05).

Assessment of chloropicrin for the control of *R. necatrix* in avocado plants

Detection of R. necatrix *in the soil using PCR confirmation assay*

PCR confirmation assays showed that the soil was successfully infected with *R. necatrix*. The expected band size was approximately 500 bp using species-specific primers for *R. necatrix* and was present in all the infected samples. Since both the untreated control and chloropicrin treated soil were infected with *R. necatrix*, the PCR assay could not distinguish between DNA obtained from viable and those from dead fungal material, since *R. necatrix* DNA was present in all the samples (Figure 3.15). The symptoms observed in the plants were due to *R. necatrix*.

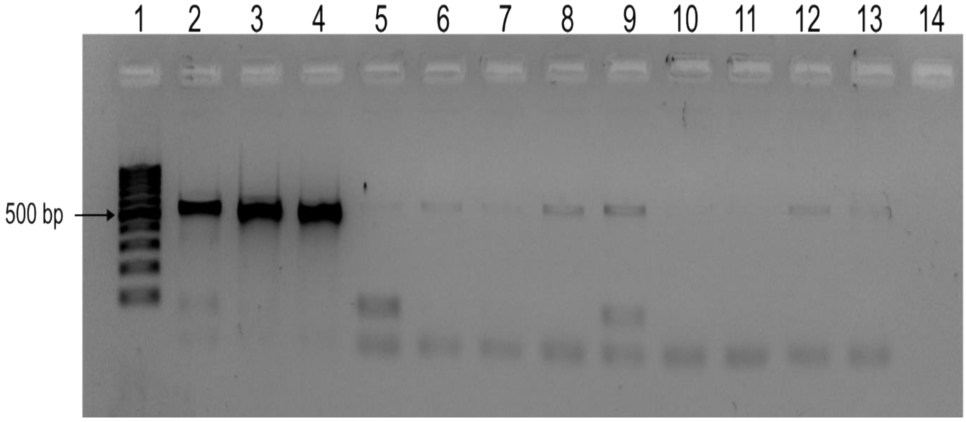

Figure 3.15: PCR confirmation assays of *Rosellinia necatrix* of untreated and treated soil with chloropicrin. The presence of a ~500 bp band confirms the presence of *R. necatrix* DNA. 1. 100 bp molecular marker; 2-4. Untreated control samples; 5-13. Chloropicrin treated samples and 14. Non-template control.

Detection of R. necatrix *in soil samples using the baiting method to determine the efficacy of chloropicrin*

R. necatrix white mycelia colonized the twigs and leaves used to bait the untreated control soil samples (Figure 3.16A). No mycelial growth on plant material used to bait the chloropicrin treated soil samples was observed (Figure 3.16B). Based on the results of this baiting study, we confirmed that no viable *R. necatrix* was present in the soil following chloropicrin treatment of the soil.

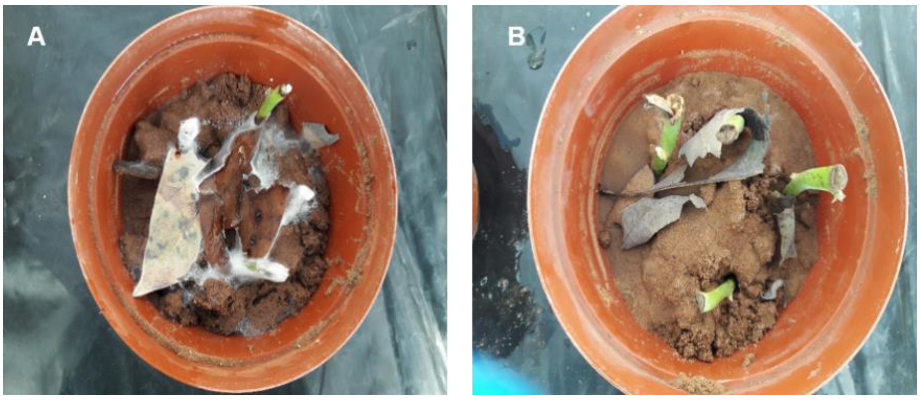

Figure 3.16: Soil was baited with avocado twigs and leaves to isolate *Rosellinia necatrix* from untreated (A) and soil treated with chloropicrin (B). White mycelia from untreated soil were observed on avocado twigs and leaves while no mycelial growth was observed from the treated soil after 30 days.

Assessment of WRR symptoms on plants grown in untreated and treated soil with chloropicrin

The first signs of stress appeared at 40 days post-planting, visible as leaf wilting of plants from untreated soil, while plants grown in the chloropicrin treated soil demonstrated no signs of leaf wilting. When the trial ended at 60 days post-planting, plants from untreated soil showed severe symptoms of leaf wilting and desiccation (Figure 3.17A (A)), while plants from treated soil appeared healthy with less leaf abscission (Figure 3.17A (B)). The disease severity was significantly less (10%) in plants grown in chloropicrin treated soil from leaf drop and drooping of only a few leaves while those grown in untreated soil demonstrated disease severity above 80% when the trial ended (Figure 3.17B).

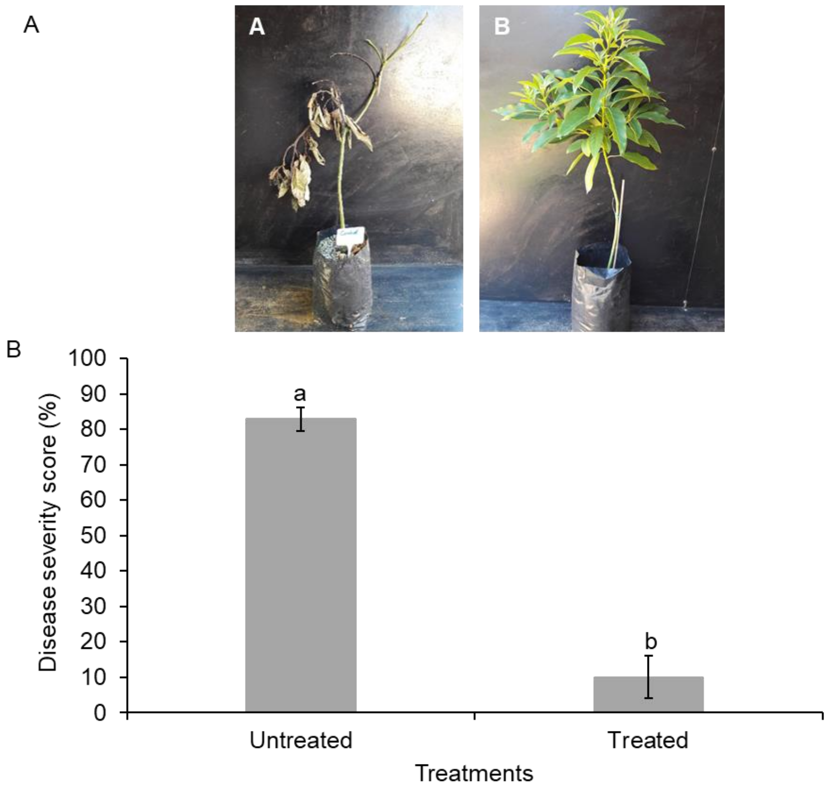

Figure 3.17: Visual assessment of aerial White Root Rot symptoms in plants grown from untreated and treated soil with chloropicrin. (A) The plants grown in untreated soil demonstrated severe leaf wilting 60 days after planting, while plants grown in chloropicrin treated soil showed less signs of stress symptoms. (B) Percentage disease severity for aerial symptoms on plants. Bars indicate standard errors of the mean of 10 replicates and means with the same letter were not significantly different according to Tukey's LSD test ($P<0.05$).

At 60 days post-planting, roots from untreated and treated soil with chloropicrin were assessed for WRR symptoms. Plant roots from untreated control soil were necrotic, rotten, and covered with white mycelial growth (Figure 3.18A (A)). Plant roots from chloropicrin treated soil had no mycelial growth and had fewer black roots (Figure 3.18A (B)). The roots from untreated soil control treatment had a significantly higher disease severity (>80%) than those from chloropicrin treated soil which showed less than 15% disease severity from black roots but not necrotic and no traces of white mycelia were observed (Figure 3.18B).

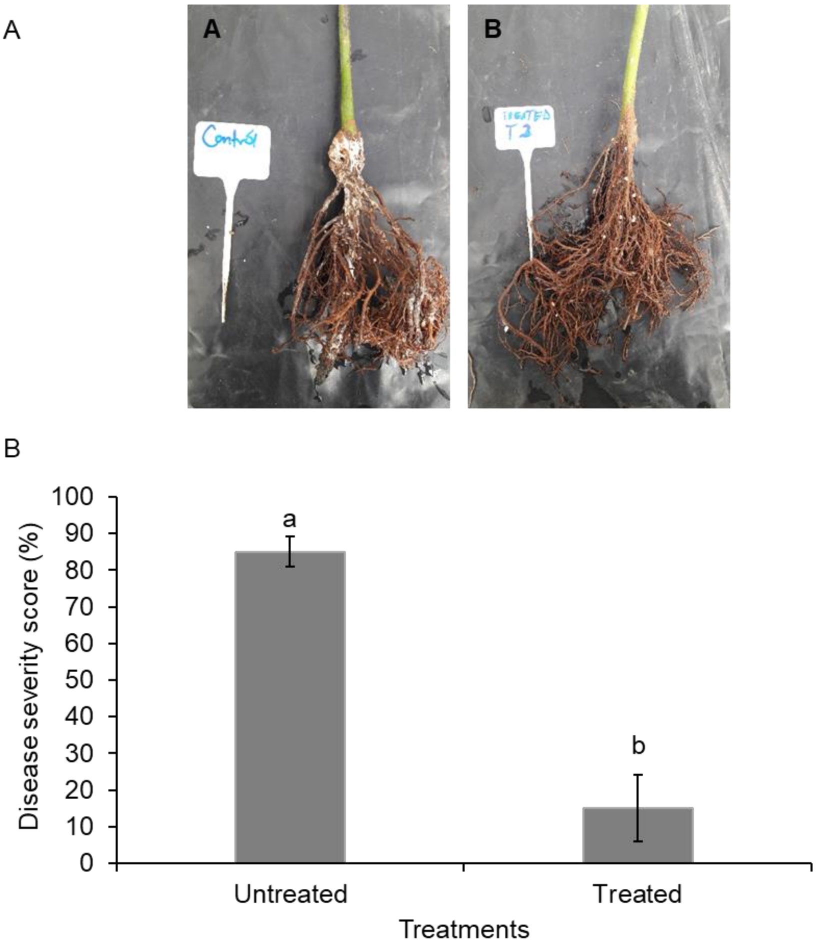

Figure 3.18: Visual assessment of White Root Rot symptoms on the roots of plants grown in untreated and treated soil with chloropicrin. (A) White mycelial growth was observed on the roots of untreated control plants 60 days after planting, while plants planted in the chloropicrin treated soil showed fewer black roots. (B) Percentage disease severity for root symptoms. Bars indicate standard errors of the mean of 10 replicates and means with the same letter were not significantly different according to Tukey's LSD test (P<0.05).

Discussion

This study showed the capacity of BCAs and chemical control products as possible treatments for plants infected with *R. necatrix*. In previous studies, BCAs of *Trichoderma* (Ruano-Rosa et al. 2010, Ruano-Rosa et al. 2014b) and rhizobacteria (*Pseudomonas* and *Bacillus* genera) (Cazorla et al. 2006, González-Sánchez et al. 2010) species were isolated from avocado roots and rhizosphere in areas infected with *R. necatrix* and selected for biological control. These BCAs were selected based on their *in vitro* and *in vivo* antagonism against *R. necatrix*. However, neither of these studies tested commercial products of BCAs against *R. necatrix*. Several successful attempts for the chemical control against *R. necatrix* in avocado were conducted in Spain and FLU inhibition of the pathogen was consistent in all the trials (López-Herrera and Zea-Bonilla 2007). FLU is not registered as a potential control for *R. necatrix* on avocado in South Africa and it is still being tested against this pathogen. Therefore, in the current study, commercial products of BCAs and FLU were selected for the control of *R. necatrix* in both *in vitro* and *in vivo* trials. Also, chloropicrin was tested to aid in understanding soil fumigation as a possible strategy against *R. necatrix* when used as a pre-plant treatment since there are no published reports of being tested in avocado orchards.

When bacterial products were assessed for efficacy against *R. necatrix* grown *in vitro*, a maximum inhibition effect was observed for BR while MG and BB failed to inhibit the growth of the pathogen. This finding showed that BR does not kill *R. necatrix* in culture but only caused a reduction in growth of the pathogen. In a previous study, inhibition of *R. necatrix* by *B. subtilis* PCL1608 filtrates grown *in vitro* was observed and the pathogen was not killed but resulted in slower growth in culture (Ruano-Rosa et al. 2014a). The products, BB and MG failed to inhibit the growth of *R. necatrix* in culture which could be hypothesised as due to a lack of antibiotics against the fungus and a result of the complexity of the fungal cell wall as bacteria need to produce a wide variety of exoenzymes (amylase, lipase and proteases) to degrade the cell wall components of fungal species (Pliego et al. 2011).

In this study, Tricho had the highest growth rate in culture and occupied more space than *R. necatrix* within a few days. When Tricho encountered *R. necatrix*, it resulted in slower growth of the pathogen. The growth rate of *R. necatrix* at the end of the trial was 10% while a previous study reported a growth of 40% when grown *in vitro* (Ruano-Rosa et al. 2017). This analysis of inhibition was based on the competition for space in Petri plates. Another study reported that upon contact with fungi (*Sclerotinia sclerotiorum*), biological control of *T. harzianum* did not kill the pathogen but a slow growth in culture was observed (Lugtenberg et al. 2017). The antagonism of Tricho against *R. necatrix* could be hypothesised to be due to the high number of antifungal compounds produced by this BCAs when grown *in vitro* (Ruano-Rosa et al. 2014a).

An *in vitro* setting is essential to ensure the selection of potential BCAs for control of plant diseases (Pliego et al. 2011). However, in the present study, all BCAs were further evaluated for their efficacy against *R. necatrix in planta* under greenhouse conditions. Plants treated with BR showed reductions in WRR symptoms, whereas plants treated with BB and MG were severely affected by the disease as the plants wilted and desiccated. The lack of suppression of WRR showed by BB and MG products when compared with infected control plants could be due to a lack of the production of antifungal compounds for efficient biocontrol (Ruano-Rosa et al. 2014a). The ability of BR to suppress *R. necatrix* could occur due to the high number of antifungal compounds produced by *B. subtilis* strains which has been previously reported (Ruano-Rosa et al. 2014b). Plants treated with BR-post treatment showed that once WRR symptoms rapidly progressed in plants, they became vulnerable, and the product failed to counteract the disease. This finding showed that application of BR as a BCA before *R. necatrix* infection plays a vital role in suppressing the pathogen by promoting growth and development of the roots prior to the introduction of the pathogen. This suggests that it could be a valuable pre-plant treatment. The product of BR contained *B. subtilis* HC8 (Table 3.1), and Ruano-Rosa et al. (2014b) reported that *B. subtilis* PCL 1608 showed satisfactory control of WRR disease when applied alone or combined with another bacteria, which agrees with other studies (Cazorla et al. 2006, Calderón et al. 2013).

The present study further showed that Tricho suppresses *R. necatrix* when applied before infection under greenhouse conditions. Similar results to this finding showed that when *T. harzianum* T22 was used to control cucumber mosaic virus (CMV) in tomato plants, disease symptoms were reduced (Vitti et al. 2016). In the current study, plants treated with Tricho showed less leaf drop and less severe wilting while WRR rapidly progressed in untreated infected controls showing severe above-ground symptoms. The effects of Tricho have been reported to increase plant growth in stress conditions due to antipathogenic activity and directly through the production of phytohormones (Harman 2000), improved solubilization and uptake of soil nutrients (Sofo et al. 2012, Stewart and Hill 2014). The colonization ability of Tricho offers better disease control in niches that cannot be exploited by chemical treatments (Ruano-Rosa et al. 2017). BCAs colonize competitively through a variety of antagonistic mechanisms and thus avoid the development of resistance in the pathogen that so often renders fungicides obsolete (Ruano-Rosa et al. 2017).

When WRR progressed, Tricho and BR products prevented a severe reduction in dry mass production and green photosynthetic tissues in treated plants. In a previous study, they observed that the effect of *T. harzianum* T22 also reduced the spread of CMV symptoms and prevented a decrease in green photosynthetic tissues (Silveira et al. 2015). The failure of BB and MG to suppress WRR in avocado caused a reduction in carbon production because the plants failed to fix carbon due to a decrease in A_N. The effect could be a result of necrotic and rotten roots due to WRR, which affected water and nutrient uptake by the plants (Benítez et al. 2004). This also negatively affected the re-allocation of different physiological activities from vegetative growth toward defence activation (Gruner et al. 2013). The decrease in dry mass of infected control plants changed carbon allocation to the shoots since most of the roots were necrotic and rotten. The treatments demonstrated variation in dry mass values, but more dry mass was allocated to the shoots than the roots. In contrast, plants under stress conditions often decreased their growth rate and dry mass production, which contributed more dry mass to the roots, to maintain a higher R:S ratio for regeneration of new roots (Yin et al. 2005).

R. necatrix treatment caused reduced water uptake by the roots as demonstrated by stomatal closure to maintain leaf water potential and physiological processes. The effect resulted in lower values of A_N in infected control plants, as consequences of stomatal closure, manifested by reduced g_s. In the present study, untreated infected control plants showed a significant decline in A_N at 56 days relative to plants treated with all the treatments when visible wilt symptoms of the leaves were apparent. This finding agrees with Zumaquero et al. (2019) where plants infected with *R. necatrix* showed a significant decrease in A_N when severe visible wilt symptoms of the leaves become evident. However, plants treated with BR and Tricho avoided stomatal closure and sustained A_N during *R. necatrix* infection. The treated plants and uninfected control plants showed ability to sustain A_N rate when compared to untreated infected control plants. This suggests that the treatments allowed plants to avoid stomatal limitation as WRR development progressed rapidly of infected control plants and such behaviour in plants has been previously reported (Martínez-Ferri et al. 2016, Zumaquero et al. 2019). This result agrees with the evidence that BCAs, especially Tricho, have the potential to increase A_N rates and the efficiency in plants (Vargas et al. 2009, Mastouri et al. 2010, Vitti et al. 2016), mainly through redox status of the plant (Harman 2011). Plants treated with BR and Tricho further avoided a decline in iWUE due to stomatal opening relative to infected control plants which had their stomata closed. The iWUE showed the growth and health status of the plants under water deficit condition, which was dependent on the amount of water used for growth and dry mass production as reported by Monclus et al. (2006).

The findings of the different treatments examined in the present study, further showed the efficacy of FLU in inhibiting *R. necatrix* both *in vitro* and *in vivo*. FLU is a broad-spectrum fungicide regularly applied in the soil, given its protective systemic or curative effects against many pathogens (Sugimoto 2002). Over the years several fungicides have been tested against *R. necatrix* which have included benomyl, carbendazim and thiophanate methyl but FLU has shown better inhibition of the pathogen (Saccas 1956, Behdad 1976, Ruano-Rosa et al. 2017, Arjona-López et al. 2020). FLU had shown higher persistence in culture than the other fungicides (López-Herrera and Zea-Bonilla 2007). In the present study, the evidence of FLU efficacy against *R. necatrix* grown *in vitro* agrees with findings by López-Herrera and Zea-Bonilla (2007). All the concentrations (0.05, 0.1 and 0.15 µg/mL) used in this study inhibited the growth of the pathogen. Recently, a minimum concentration of 0.01 µg/mL of FLU in culture completely inhibited mycelial growth of nine isolates of *R. necatrix* (Ruano-Rosa et al. 2017), and the value was 10 times less than the present study and a previous study by López-Herrera and Zea-Bonilla (2007). This suggests that FLU inhibits the pathogen at a minimum concentration in culture, however this might be different under greenhouse and field conditions.

This study further showed that FLU reduces WRR symptoms, and plants remained healthy for more than a month since the last application. These results agree with López-Herrera and Zea-Bonilla (2007), who reported that FLU reduced WRR disease incidence and plants remained healthy for more than two months after the second infection with *R. necatrix*. Previous studies suggest that higher persistence of FLU in the soil is due to residual activity of the fungicide which is about one year (López-Herrera and Zea-Bonilla 2007, Arjona-López et al. 2020) when compared with thiophanate methyl residual activity which is only four months (Kanadani 1998), while carbendazim is six months (Sharma and Gupta 1985). The ability of the fungicide to reduce inoculum load of *R. necatrix* in the soil could be due to solubility, chemical reactions in the soil and contact with the root system of the plants (Shukla et al. 1972). Recently, FLU was applied at 0.3 m depth around infected avocado plants and demonstrated a reduction in inoculum load of *R. necatrix* in the soil when compared to untreated soil (Arjona-López et al. 2020). Reducing the progression of *R. necatrix* inoculum in the soil depends on the concentration of the fungicide and depth of application. This was possible using an injector to apply the FLU in the soil around a diseased plant repeatedly to reduce the *R. necatrix* inoculum load (Arjona-López et al. 2020)

Among the chemical products, chloropicrin demonstrated the highest efficacy of *R. necatrix* control when compared to FLU and the BCAs. Chloropicrin is a soil fumigant used to control soil-borne pathogens (Enebak et al. 1990, Ajwa et al. 2010). The current study showed that chloropicrin completely inhibited *R. necatrix* grown *in vitro* and under greenhouse conditions. The soil fumigant showed potential as a strategy of control against the pathogen. In this study, chloropicrin killed *R. necatrix* when tested as a pre-plant treatment under greenhouse conditions. The soil fumigant showed to be highly effective in reducing WRR disease incidence in plants throughout the trial period. This finding agrees with previous studies where they reported the efficacy of chloropicrin in reducing soil pests, including insects, fungi, nematodes, bacteria, and weeds (Ajwa et al. 2010, Dangi et al. 2015). In another study, the soil fumigant was used to control laminated root rot caused by *Phellinus weirri* (Murr) Gilb., by injection into infected stumps of Douglas-fir seedlings (*Pseudotsuga menziesii*) and it killed the fungus in the roots (Massicotte et al. 1998). However, non-target beneficial organisms are unavoidable when chloropicrin is used to control pathogenic microorganisms and it causes a shift in microbial community composition in the soil (Dangi et al. 2015, Li et al. 2017). It is not recommended to apply chloropicrin in the orchards with trees because it will have negative effects on the roots as it has been reported previously to diffuse slowly into the roots and kill the plants (Haar et al. 2003). Therefore, it is recommended to apply chloropicrin at pre-planting and incorporate beneficial microorganisms like *Trichoderma* in the soil following soil fumigation to improve the soil microbial community structure (Dangi et al. 2015). The efficacy of chloropicrin to reduce the pathogen load in the soil before planting can lead to success in controlling WRR.

This study had several limitations which can be avoided in the next investigation of WRR control. Following treatment using BCAs and FLU, the amount of inoculum load in the soil was not quantified to estimate the effect of the control products against *R. necatrix*. Additionally, the presence of soil microbial communities was not tested following the soil fumigation process in the greenhouse. However, previous studies suggested that given enough time, the soil microbiota composition can eventually return to its original state after fumigation (Dangi et al. 2015, Li et al. 2017).

Conclusion

In this study, BCAs and chemical control products were investigated *in vitro* and *in vivo* against *R. necatrix*. The inhibition effects of BCAs (BR and Tricho) grown *in vitro* showed potential control against *R. necatrix*. The results of chemical products (FLU and chloropicrin) showed maximum inhibition of *R. necatrix* grown *in vitro*. The greenhouse trial showed that plants treated with Tricho and BR had less WRR symptoms when compared to infected control plants. FLU suppressed WRR disease progression when applied after infecting the plants with the pathogen. Chloropicrin showed better control of *R. necatrix* in comparison with the BCAs and FLU, but as a soil treatment.

Infected control plants further showed a decrease in A_N, g_s and iWUE compared to all the treatments as a result of stomatal limitation. Plants treated with BR, Tricho and FLU were able to sustain dry mass production and A_N during WRR infection when compared to infected control plants. Reductions in these leaf gas parameters in infected control plants and other treatments gave a better indication of plant stress and exposed the effects of the pathogen when plants were treated with BCAs and chemical products. The ability of treated plants to maintain stomatal opening is a result of the generation of new roots and carbon partitioning to important plant organs during WRR infection.

This study suggests the possible use of BCAs and chemical products to provide an integrated management approach to control *R. necatrix*. This work also advances our knowledge of how BCAs influence plant physiological responses during pathogen stress. Therefore, the next study will be in the field and will assess the changes in the rhizosphere microbial communities to explore the effects of soil fumigation, FLU, and biological treatments on plant growth. Also, a metagenomics and transcriptomics will offer a powerful approach for investigating the effects of fumigation on soil bacterial functional diversity in avocado orchards. This approach will detect the shift in microbial community composition in the soil because of fumigation which can lead to changes in functional diversity of microorganisms and soil quality. This study contributed knowledge about the potential control products of *R. necatrix* in the absence of registered products for the control of this pathogen in avocado orchards, specifically in South Africa.

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CHAPTER 4

General conclusion and recommendations

Introduction

The avocado industry has increased enormously over the years in South Africa, with approximately 17,500 ha of commercial avocado orchards. The total production was estimated at 170,000 Mg in 2018, of which approximately 86,000 Mg were exported, mainly to Europe and the United Kingdom (SAAGA 2019). However, avocado production is challenged by several pests and diseases. Two important root rot diseases are Phytophthora Root Rot (PRR) and White Root Rot (WRR). PRR is currently effectively managed by implementation of an integrated management approach including the use of partially resistant rootstocks such as ‘Dusa®’ and phosphite treatments. In contrast, WRR caused by *R. necatrix* can infect *P. cinnamomi* partially resistant or tolerant rootstocks, thus, the disease poses a threat to the avocado industry in South Africa. Since the identification of this pathogen by van den Berg et al. (2018), several incidences are still continuously reported to the Avocado Research Programme, University of Pretoria, South Africa. As of 2019, 29 avocado orchards in Limpopo, Mpumalanga and Kwa-Zulu Natal have been tested for *R. necatrix* using PCR confirmation assays and 16 samples tested positive for the pathogen (Hartley 2020).

In order to understand the effects of *R. necatrix* in different avocado rootstocks, it was important to analyse the physiological response of the plants during WRR disease progression in a greenhouse trial (Chapter 2). Physiological responses are important indicators of plant stress during infection by root pathogens and they can detect changes in plant growth before above-ground symptoms are evident. The results showed that ‘Dusa®’ (industry rootstock) is highly susceptible to *R. necatrix* and there was no significant difference when compared to R0.38 and R0.06 rootstocks. All the rootstocks demonstrated above-ground symptoms as wilting of the leaves at 35 dpi and more than 90% of the plants were confirmed dead within 60 days. Roots of infected plants were black, necrotic, and covered with white mycelia for all the rootstocks. This suggests that all rootstocks used in this study are highly susceptible to WRR.

The significant decrease in A_N and g_s of infected plants matched with the first visible symptom of leaf wilt for all rootstocks. In contrast, F_v/F_m ratio of infected plants showed a significant decrease when compared to control plants of all the rootstocks before above-ground symptoms were evident. This suggests that F_v/F_m ratio did not match with the appearance of leaf wilting symptoms. Results of F_v/F_m ratio indicated that the pathogen reduces photosynthetic rate and intercellular CO_2 concentration in the leaves of infected plants during the pre-symptomatic stage. The dry mass of infected plants was substantially reduced relative to control plants showing evidence of the effects of the pathogen. This indicated that as WRR progressed rapidly, plants closed their stomata which limited CO_2 diffusion into the leaves, resulting in reduced carbon fixation of infected plants.

This study provided an insight into the change in fluorescence and leaf gas exchange caused by WRR infection. These parameters are of significance as they have potential to be used to understand the onset of stress and WRR disease progression in plants. The pathogenicity data provided consistent disease severity, which was matched with the changes in physiological parameters of the plants as WRR progressed rapidly. Therefore, the study provided a reliable method for detecting of *R. necatrix* in plants under greenhouse conditions.

The assessment of biological control agents (BCAs) and chemical products against *R. necatrix* were evaluated in the laboratory and under greenhouse conditions (Chapter 3). This study provided the required information to understand the potential of these control products against *R. necatrix* in avocado. The laboratory assays showed that *Trichoderma* and B-Rus resulted in substantial growth reduction of *R. necatrix* in culture. The chemical product, fluazinam substantially inhibited the growth of the pathogen at all concentrations. The use of chloropicrin resulted in non-viable mycelial growth following treatment with the product. These results suggest that BCAs (*Trichoderma* and B-Rus) and chemical products (fluazinam and chloropicrin) can reduce *R. necatrix* growth under laboratory conditions. Therefore, these products were further tested in greenhouse trials to confirm their potential to control *R. necatrix.*

In a greenhouse trial, the application of *Trichoderma* and B-Rus before infection with *R. necatrix* showed a significant decrease in WRR symptoms of the plants. The application of fluazinam after infection resulted in reduction of WRR progression.

Plants treated with these BCAs and fluazinam demonstrated less leaf drop and they were still healthy at the end of the trial when compared to infected control plants. Most importantly, the treated plants showed sustained photosynthesis ability and their dry mass was significantly higher when compared to infected control plants. However, viable *R. necatrix* was re-isolated from this treatment and suggested that the pathogen was not killed during the trial. Pre-treatment of the soil with chloropicrin before planting killed the pathogen as non-viable mycelia were observed using a baiting method. Plants from the soil treated with chloropicrin did not demonstrate any signs of infection and no white mycelium was observed on the roots.

The study has provided additional information about the effects of *Trichoderma* and B-Rus, and chemical products (chloropicrin and fluazinam) against *R. necatrix* in avocado. These products have the potential to control the spread of WRR in avocado orchards. The present data should be complimented with field trials to shed light on the effects of chloropicrin, fluazinam, and BCAs (*Trichoderma* and B-Rus) on the soil microbial community in avocado orchards.

The present study has provided useful information of potential control of WRR to avocado researchers and the industry. The physiological response of avocado to *R. necatrix* data provided insight into disease monitoring which could be adopted at field level using remote sensing data. From the present study, physiological changes in g_s and flouerescence can be used in combination with remote sensing to identify stressed trees at field level. The BCAs and chemical control products data provided more detailed information on the products available to control WRR disease in avocado orchards. Additionally, this study has provided necessary information that can aid in the registration of control products for WRR in avocado. Finally, the results revealed that an integrated approach using a combination of detection techniques of the pathogen, BCAs, and chemical control products to control WRR, can successfully be implemented in avocado orchards.

Recommendation for Future Research

The present study investigated detection techniques and assessed BCAs and chemical products against *R. necatrix* under laboratory and greenhouse conditions. Further research is recommended to continue improving early detection techniques and to assess BCAs (*Trichoderma* and B-Rus) and chemical products (chloropicrin, fluazinam) against *R. necatrix* under field conditions. Future field trials are recommended to measure microbial community changes in the rhizosphere and to explore the effects of BCAs, fluazinam and chloropicrin fumigation on plant growth and soil health. The bigger picture is to have 'Big data' that can be available to farmers for an integrated management approach of *R. necatrix* and to establish more collaboration to advance research focused on this pathogen. Field trials will also assist in the registration of control products like fluazinam and chloropicrin for the control of WRR in avocado orchard.

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