Assessing the threat of the polyphagous shot hole borer ambrosia beetle and its fungal symbiont – *Fusarium euwallaceae*, to *Persea americana*, in South Africa

By

Michael du Toit (14003253)

Supervisor: Prof. Noëlani van den Berg

Co-supervisors: Prof. Wilhelm de Beer

Dr Gerda Fourie

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Submitted in fulfilment of the requirements for the degree of

MSc Microbiology

Department of Biochemistry, Microbiology and Genetics

Division of Microbiology

2019

PREFACE

The basis of this research stems from the recent introduction of the internationally invasive pest complex between the ambrosia beetle: *Euwallacea fornicatus* and its fungal symbiont, *Fusarium euwallaceae,* into South Africa. This pest complex causes Fusarium dieback (FD) on a large variety of plant species and the arrival and spread of this pest has become of concern to local agriculture, particularly the avocado growing communities. **Chapter 1** of this study summarises the international occurrences and impact of this invasive pest species and other ambrosia beetles. The current understanding of diseases associated with ambrosia beetles and available control and management approaches are also discussed. During the research conducted in **Chapter 2**, commercial avocado orchards and residential avocado trees were sampled for the presence of wood-boring beetles and fungi associated with their damage, to determine the distribution and prevalence of the beetle and its associated fungal organisms, specifically on avocado. This involved the collection of various beetles and isolation of associated fungi, causing damage on avocado as well as the detection of the causal agent of FD (*Fusarium euwallaceae*), from diseased avocado tissue, in South Africa. Further investigations were performed to verify the identity of the isolate and its pathogenicity, to avocado. In addition to persistent monitoring and surveying for the presence of *E. fornicatus* and its symbionts, a pathogenicity study was conducted on the susceptibility of two avocado fruit cultivars to the fungal symbionts of *E. fornicatus* (*F. euwallaceae* and *Graphium euwallaceae)*. This was followed by investigating several chemical and biological control agents for their effectivity against *F. euwallaceae* mycelia, towards achieving complete mycelial inhibition, *in vitro,* in **Chapter 3**. This thesis is concluded (**Chapter 4**) by recommending future research ideas and changes to research protocols.

Chapter 1: The beetle-fungus interaction, causing Fusarium Dieback on *Persea americana*

TABLE OF CONTENTS

INTRODUCTION

The emergence of new diseases, within an area, is a routine event in the evolutionary ecology of pathogen populations (Cleaveland *et al.*, 2007). Pathogen populations experience accelerated geographical shifts, as a result of rapid globalisation and increased anthropogenic influence (Crowl *et al.*, 2008). In the case where sessile host organisms, such as plants, are involved, the influence of this routine pathogen spread is highly meaningful. The only natural defences, that plants have against pathogens, are products of the evolutionary arms race that they have endured with pathogens they have co-evolved with (Stahl & Bishop, 2000). This means that plant species, that have not been exposed to certain pathogens, will lack the appropriate defence pathways necessary for resistance.

One major element that accounts for the emergence of new pathogens, on a global scale, is the introduction of pathogen populations, through vector organisms, usually in the form of insects (Sisterson, 2008). Insect vectors offer accelerated spread to pathogen populations and can effectively introduce a disease into a new area, despite proper management techniques, employed by conventional practices (Altieri & Nicholls, 2004). Unlike diseases that have been present within an area, emergent diseases are often able to present with the element of surprise. The variation in environment and climate allows new diseases to inhabit host organisms that they have not affected before, as well as presenting levels of virulence, which may be significantly different from what is known in their native areas. In this way, pathogens that are spread through insect vectors, are considered to be important emerging

diseases (Mayer *et al.*, 2008), particularly when they are introduced into an area that they have not been present before.

A group of insect vector organisms, that has posed a threat to international agriculture and forestry biosecurity, are ambrosia and bark beetles (Eskalen *et al.*, 2013). Certain ambrosia and bark beetles act as insect vectors, that penetrate plant-host tissue and inoculate ambrosial fungi into live host plants, from specialised fungus-cultivating organs, known as mycangia (Coyle *et al.*, 2005). These ambrosia beetles and the fungi that they carry, exhibit mutualistic symbioses, whereby the fungi rely on the beetles as their vectors, to inoculate and spread within an area, as well as reaching new areas. The mycophagous, or fungal-feeding beetles, in turn, rely on the colonisation and growth of their symbiotic fungal partners, as a source of nutrition, as these beetles are mostly unable to digest healthy plant tissue (Beaver *et al.*, 1989).

The polyphagous shot hole borer (PSHB) or *Euwallacea fornicatus* Eichhoff. is one such an ambrosia beetle, originally from South-East Asia, that has a symbiotic relationship with several fungal organisms (Freeman *et al.*, 2013). This ambrosia beetle can cause extensive damage, to a large variety of trees, by acting as a vector to inoculate pathogenic fungi into reproductive and non-reproductive hosts, causing Fusarium Dieback (Eskalen *et al.*, 2012). This invasive beetle and its fungal symbiont, specifically *Fusarium euwallaceae,* phylogenetically positioned in the *Fusarium solani* species complex – FSSC (O'Donnell *et al.*, 2015), forms a complex that is internationally notorious for the damage it has caused to trees, specifically to avocado (*Persea americana* Mill.), in Israel (Mendel *et al.*, 2012) and the United States of America (Eskalen *et al.*, 2012, Umeda *et al.*, 2016).

The threat, posed by ambrosia beetles and their accompanying fungi, is not considered a new topic, within the scientific community. However, when these beetles are found in their non-native distributions, they can cause considerable levels of damage to plants, where agricultural practices are unconditioned to dealing with their anomalous disease lifestyles (Kühnholz *et al.*, 2001). As such, management and control practices are often very difficult to devise and effective treatment may be unfeasibly expensive or take a great deal of time to develop. The implication hereof is that a large portion of basic research must be conducted, to understand the fundamental interactions between the host, vector and the accompanying fungal pathogen, towards establishing a sustainable and meticulous management practice.

THE EFFECT OF GLOBALISATION ON PATHOGEN SPREAD AND BIOSECURITY

Globalisation and the rapid development of interdependence between countries result in large-scale movement of biological components that would not have occurred naturally (Bright, 1999). As such, the presence of alien plant pests and pathogens are rapidly increasing in many countries as an implication of increasing trade. This is noteworthy, as it is commonplace that the threat to biosecurity, posed by emerging pathogenic micro-organisms, is overlooked, specifically from a border-security perspective. The spread of pathogens is oftentimes regarded as an inevitable consequence of globalisation (Perrings *et al.*, 2005).

Increased trade and anthropogenic movement across the planet means that humans, animals and plants are regularly exposed to new environments and pathogens

(Perrings *et al.*, 2005). The threat posed by these new or emerging diseases has far-reaching effects, including human, animal and plant health, biodiversity, as well as food biosecurity. Food biosecurity, as far as plant-based food sources are concerned, is perhaps one of the most deliberated topics in the information era (Dobson *et al.*, 2013). Emerging plant diseases act as continuous sources of pressure for the total collapse of the international agricultural industry unless farming strategies are monitored and optimised (Gullino *et al.*, 2008) or adapted and better management practices are consistently implemented.

DISEASES CAUSED BY VECTOR-BORNE PATHOGENS

Pathogen spread occurs via many routes, but perhaps the most responsible biological entity is vector-assisted pathogen spread (Purcell, 1982). Vector organisms can differ considerably from each other but do not generally cause disease themselves (Carter, 1962), instead, they spread disease by conveying pathogens from one host or location to another. Some of the most well-known vectors, responsible for economic and societal damage, are invertebrate vectors, particularly insects (Frago *et al.*, 2012). Insect pathogen-vectors boast an enormous level of diversity, encompassing members from nearly all insect orders. They are also capable of, and responsible for, dispersing vast numbers of pathogenic micro-organisms. They have been connected with the spread of viruses, bacteria, fungi, and various parasites, like nematodes and protozoans (Leach, 1940, Harris & Maramorosch, 2013).

Some of the most notorious insect vectors associated with human health are mosquitos, ticks, lice and flies (Lounibos, 2002). Vector-borne, human diseases

account for more than 17% of all infectious diseases worldwide, causing more than 700 000 deaths annually (WHO – World Health Organization, 2019), by transmitting diseases like malaria (Macdonald, 1957), Zika (Petersen *et al.*, 2016), Lyme disease, Typhus and African trypanosomiasis (Gubler, 1998). Similarly, vector-borne animal diseases, transmitted by flies, midges and fleas are also responsible for threatening animal life, as well as causing substantial economic damage, by causing African horse sickness and the plague (Mellor & Hamblin, 2004), to name but a few. The hazard of insect vectors is often considered only a threat to humans and animals, but this is certainly not the case. Many of the most devastating vector-borne diseases are associated with and cause diseases on important plant species only (Perrings *et al.*, 2005). Some of the most widely recognised plant pathogenic vectors include mealybugs, treehoppers and aphids, which are capable of harbouring and transmitting a plethora of different plant viruses (Ingwell *et al.*, 2012). Several different fly species are also frequently associated with the transmission of a broad variety of pathogenic micro-organisms (Rogers, 1989), responsible for the causation of disease on almost all forms of plant life.

Perhaps the most significant of these plant pathogenic vectors belong to the largest insect order: Coleoptera, consisting of the beetles (Crowson, 2013). Most beetles fill crucial and beneficial roles within just about all natural systems, by acting as important scavengers and decomposers (Crowl *et al.*, 2008). Many beetles are directly involved in the biological recycling of organic nutrients within an ecosystem, along with several other biotic and abiotic factors. In addition, many predatory beetle species, such as ladybugs, occupy invaluable niches as natural biological control agents that limit the abundance of aphids, scale insects and other agricultural pests (Scudder, 2009). It is

therefore important to consider that only a limited portion of beetle species are associated with plant diseases (Rust & Su, 2012).

AMBROSIA BEETLES – GENTLE FARMERS OR DESTRUCTIVE PATHOGEN VECTORS

Another important group of beetles, normally involved as nutrient recyclers, are the ambrosia beetles (Borden, 1988). This otherwise un-invasive and gentle group of insects has been consistently associated with mutualistic symbioses, between itself and several fungal organisms, as an adaptation for efficient biomass decomposition (Borden, 1988). It is evident that these organisms have evolved in tandem with one another, probably the most obvious evidence is the degree of refinement showcased within the interaction between the beetles and their fungal partners (Purcell *et al.*, 2012). The beetles have evolved highly-efficient sustaining and cultivation techniques to effectively transport and house their fungal partners (Six, 2003). They do so by cultivating one or more fungal partners within a specialised housing organ, known as a mycangium (Toki *et al.*, 2012). The mycangia of these beetles vary widely in their form and structure, alluding to the level of evolutionary sophistication between the beetle and its associated, obligate symbionts (Six, 2012). The mycangia range from internal to external mycangia as well as in their structure and location in and on different areas of the insect's body (Six, 2012). Some of these different mycangial structures are illustrated in **Figure 1**.

1.

2.

3.

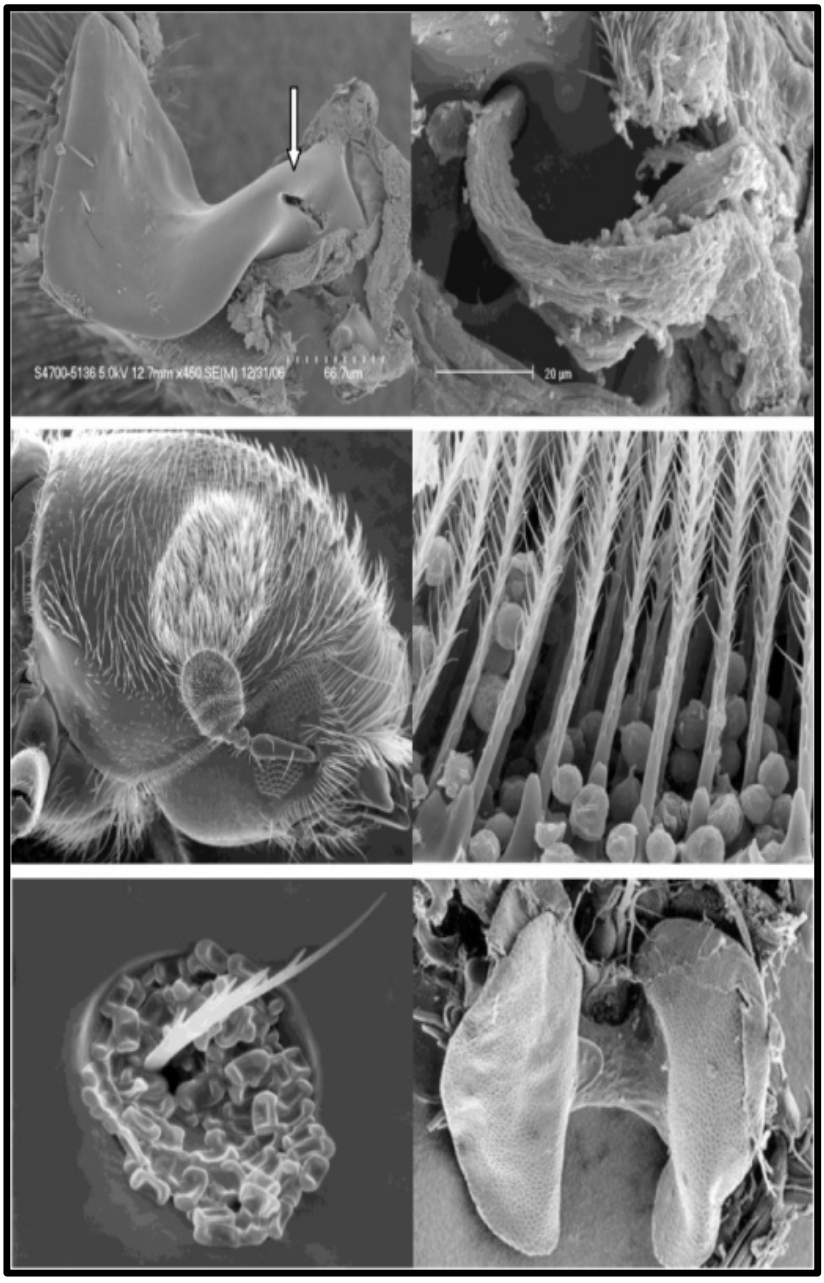


Figure 1: Examples of different mycangial structures, found in and on various parts of ambrosia beetles. Mycangia take on many forms of specialisation including simple pocket structures known as sac mycangia (shown with an arrow on the first image, first row and the close-up beside it). Below the sac mycangium, the oval brush mycangium on a female as well as the close up of the brush mycangium can be seen (row two). In the third row of images, spores can be observed in an opening known as a pit mycangium (left) and beside that a different type, known as the paired sac mycangium (right). Images obtained from Six (2012).

The ambrosia beetles carry out their ecological role by penetrating dead, stressed and sometimes even healthy plant tissue, thereby inoculating their pathogenic and non-pathogenic fungal partners directly into the plant tissue (Carrillo *et al.*, 2012). While burrowing into the plant tissue (of a reproductive host), the adult female beetle constructs her natal galleries and lays numerous eggs (Brockerhoff *et al.*, 2006). The fungi proceed to colonise and invade the plant tissue and produce slimy spore drops to further adhere to the insect's body (Khadempour *et al.*, 2012), often causing staining within the plant host tissue. The fungal mycelia also produce swollen structures known as conidia, which serve as the primary food source (Freeman *et al.*, 2012) for the mycophagous ambrosia beetles and their larvae. In this way, the interaction between the fungus and its beetle vector is a true mutualism, in that the fungal organisms are efficiently protected and sustained within a suitable environment, while also being effectively transmitted from one host to another (Biedermann *et al.*, 2009). The beetles, in turn, depend on the fungi to avoid the consumption of toxic phytochemicals and phytohormones and rely directly on the fungal structures as a source of nutrition (Popa *et al.*, 2012). This refined symbiosis is one of the few cases, along with leafcutter ants and termites, where a non-human organism displays the ability to "farm" its own fungus, for consumption (Currie *et al.*, 1999, Aylward *et al.*, 2012).

Importantly however, these ambrosia beetles use the above strategy to decompose and recycle large amounts of plant biomass within an ecosystem and are not generally considered as serious pathogen-vectors in their native environments (Mendel *et al.*, 2012). One therefore has to accredit the threat from these beetles as a product of globalisation and increased trade, particularly the trade of plant matter. Ambrosia beetles are mostly considered agricultural pests, attacking healthy plants, outside of

their natural geographic ranges and in areas where human disturbance or climate change have altered the natural state of an ecosystem (Mendel *et al.*, 2012).

HISTORY OF DAMAGE FROM AMBROSIA BEETLES

The instances of ambrosia beetle damage must be considered as an overall threat to international agriculture biosecurity, as they have been consistently responsible for damage to healthy or mildly stressed plants. Three of the most impactful of these beetles will be discussed, to shed light as to the degree of risk they present.

The first being laurel wilt caused by the Redbay ambrosia beetle – *Xyleborus glabratus* and its fungal symbiont *Raffaelea lauricola*. Many plant pathologists are familiar with laurel wilt and the level of devastation that it has caused, in particular to the American avocado growing industry. The beetle, native to Asia, is thought to have been transported to the South-Eastern part of the United States of America, where it is considered a highly-invasive alien pest species (Ploetz *et al.*, 2012). As with many pathogenic ambrosial fungi, the general mode of action includes a vascular, systemic wilt and an impairment to the xylem function and hydraulic conductivity, once the pathogen starts to colonise the water-conducting vessels. In areas with severe infestation, up to 90% tree mortality has been observed (Inch & Ploetz, 2012). This pest complex devastates and almost entirely debilitates the ability for avocado cultivation within an area where it has established (Hughes *et al.*, 2012).

Megaplatypus mutatus and *Raffaelea santoroi* – This is by far one of the most unique interactions between an ambrosia beetle and its fungal symbiont. This pest complex is considered a serious threat to the timber industry as it attacks only healthy, standing

trees, particularly woody plant species such as poplar trees (Humble *et al.*, 2007). It has also been reported to be of great implication to many highly valuable tree species, particularly in South America, such as the greatly sought-after and rare *Caesalpinia echinata* (brazilwood) trees. The fungal member of the interaction (*R. santoroi*) is unlike many other ambrosial fungi, in that it is not considered to be pathogenic on its own, instead, it grows saprophytically causing staining of the brood gallery walls (Dolinko *et al.*, 2016). The second and third instars of the beetle larvae then proceed to feed on the growing mycelia (Dolinko *et al.*, 2016). After this period, the following larvae and beetles become xylophagous and proceed to feed directly off the woody plant tissue, throughout their development. The result of this fungal staining and direct wood consumption greatly affects the strength, structural integrity and appearance of the wood, drastically limiting its use and value (Kirkendall, 2017).

Lastly, Fusarium Dieback caused by *E. fornicatus* (Gomez *et al.*, 2018, Smith *et al.*, 2019), commonly known as the PSHB (polyphagous shot hole borer) and its primary fungal symbiont – *F. euwallaceae.* Until recently, this ambrosia beetle, as well as its fungal symbiont, have been considered somewhat of a taxonomic oddity, since they both form part of large *senu lato* species complexes (O'Donnell *et al.*, 2015). The fungal partner is included in the FSSC (*Fusarium solani* species complex), which comprises many fungal members, of which only a few have enjoyed proper taxonomic delimitation (Kasson *et al.*, 2013). The beetle, also part of a species complex, has been shown to be morphologically identical to the other members within the complex, presenting difficulties for their delineation and distinctly highlights the limitations of applying species concepts based on morphology exclusively.

Nonetheless, this beetle and its primary fungal symbiont have also been extensively linked to agricultural damage, specifically with reference to the avocado industry, in countries like the United States of America and Israel (Mendel *et al.*, 2012). In addition, a recent study – as part of a sentinel programme, conducted in South Africa, detected this ambrosia beetle-fungal complex, damaging London Plane trees (*Platanus x acerifolia)* (Paap *et al.*, 2018). These sentinel programmes aim to detect arthropod pests and agents of disease and subsequent genetic analysis proved that the internationally invasive haplotype (H33) of the beetle was present. In the time since then, the first report of *F. euwallaceae* causing necrotic lesions on *Persea americana* in South Africa was published (van den Berg *et al.*, 2019). The report describes the isolation and identification of *F. euwallaceae,* from symptomatic tissue from a backyard avocado tree, by using the translation elongation factor 1α (*TEF1*-α), β-tubulin and RNA polymerase II second largest subunit (*RBP2*) gene regions. A PSHB beetle specimen could not be retrieved from the avocado tissue, but a confirmed PSHB specimen (*COX1* gene region) was retrieved from a diseased Chinese Maple (*Acer buergerianum*) tree, approximately 2 km away, harbouring *F. euwallaceae* that was genetically identical to the isolate from the avocado tissue.

This early detection is of particular significance to the South African avocado industry, especially when considering the historical damage that this beetle has caused, directed at avocado growing and cultivation. This particular beetle-fungus interaction will be the primary focal point of this review.

FUSARIUM DIEBACK: A THREE MEMBER INTERACTION

In order to diagnose an instance of Fusarium Dieback (FD), several components need to be considered. Like all other diseases, the classic scheme necessary for a disease to occur is necessary – suitable environmental conditions, a virulent pathogen and a susceptible host (Scholthof, 2007). Another dynamic that needs to be adjoined to the above notion is that pathogens are not universally facilitated by the presence of vector organisms, therefore an extra level of interaction must be considered: the interactions between the vector and the pathogens that they harbour (Nuttall *et al.*, 2000). For that reason, one needs to consider the fundamental biotic members: the host being infected, the vector organism and the vector-borne pathogen.

a. Host range of FD and the plant host *Persea americana*

The first and most obvious component for a disease to exist, provided a suitable environment is present, is a susceptible host organism. In the case of FD, this aspect has far-reaching implications. This is because of the enormous host range that is exhibited by this disease. In a study performed by Eskalen *et al.* (2003), the authors surveyed the degree of host specificity exhibited by the pathogen and its vector. Astoundingly enough, of the 335 plant species that were included in this study, 207 (62%) plant species, representing 58 plant families, showed signs and symptoms consistent with attack by the pest complex. Out of these species that were severely affected, some of the world's most agriculturally important plants include Sweet orange (*Citrus sinensis*), Macadamia (*Macadamia integrifolia*), Grape (*Vitis vinifera*) and Avocado (*Persea americana*) (Eskalen *et al.*, 2013). For the sake of simplicity and

due to the extensive host range, one of these agriculturally important plant hosts will be the particular focus of this study.

Perhaps one of the most impacted of all the plant hosts, affected by FD, is the avocado tree (Byers & Maoz, 2017). *P. americana* is a flowering plant from the Lauraceae family (Scora & Bergh, 1989), that has, in the last couple of years, experienced a huge upwards flux in value and demand, such that it is currently considered one of the world's most valuable crops (Dávila *et al.*, 2017). In addition to the raw value, related to large international demands for avocados and avocado products, most commercial avocado fruit trees are specialised through propagation of grafting material, to resist soil-borne pathogens, obtain fruit of selected varieties/cultivars (Thorp & Sedgley, 1993) and to ensure consistent production of high quantities of fruits (Nel & De Lange, 1985). The combination of high demand and highly-valuable fruit crops means that any threat, to the local and international avocado growing industries, is approached cautiously and seriously. In areas where the pest complex has established, FD has caused extensive damage, not only to fruit crops but also to urban, ornamental as well as native plant species (Short *et al.*, 2017).

One of the most noteworthy characteristics of FD is that the disease presents symptoms that vary drastically from one host to the other (Eskalen *et al.*, 2013). The symptoms of FD are usually a combination of indicators that include, but are not limited to gumming at the wound entry point, staining of the plant tissue, the formation of sugary compounds and the presence of powdering at the point of entry (Eskalen *et al.*, 2012). On avocado, the most noticeable symptom is the presence of a white sugary exudate, more generally known as a sugar volcano, which is commonly present

during the early phases of tree colonisation **(Figure 2)**. The large quantities of white exudates are composed mainly of non-structural carbohydrates: perseitol and mannoheptulose and it is hypothesised that this is a form of general disease response elicited by the plant host (Liu *et al.*, 2002, Carrillo *et al.*, 2016). In addition to this signature symptom, very small, pinhead-shaped, entry holes are formed by the PSHB upon entry, usually found together with some frass when scraping away the sugary exudates. When removing the bark at a point of entry, a soft brown lesion can be observed, which gradually spreads as the pathogen colonises and blocks the plant's vascular tissue. Finally, when a cross-section of an infested avocado tree branch is performed, beetles and larvae can sometimes be found within the galleries and significant damage to the conducting vessels can be observed, along with the presence of some fungal staining (Eskalen *et al.*, 2012, Umeda *et al.*, 2016).

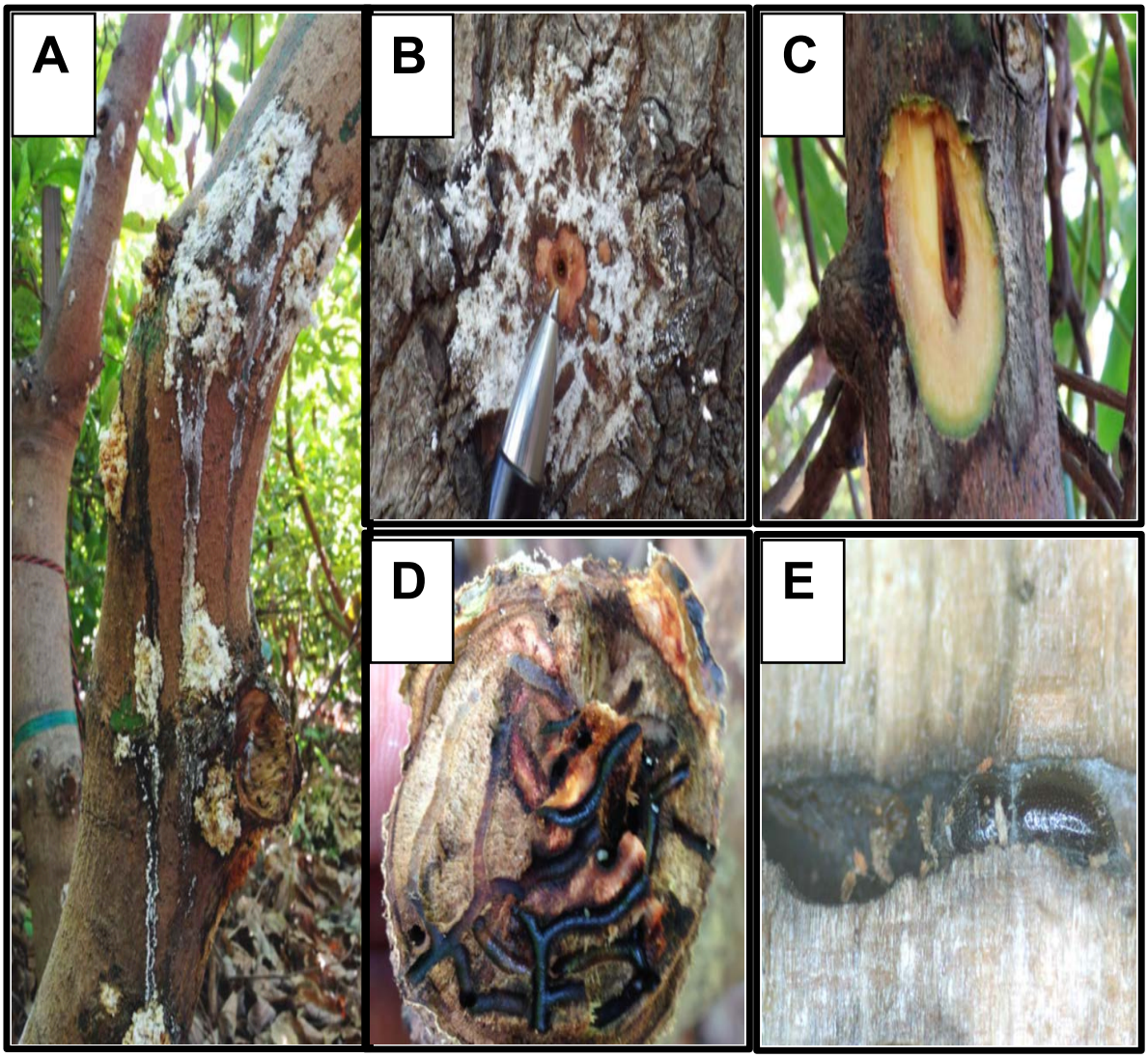


Figure 2: The symptoms of Fusarium dieback (FD) on *Persea americana.* **A** – The signature symptom of FD on avocado trees, whereby sugary exudates are expelled by an infected host. Rain often washes away this sugary debris, so it is important to note the other symptoms. **B** – Very small entry holes, formed by the beetles upon entry. **C** – Stripping away of bark from the point of entry reveals wet, brownish lesions that spread as the disease progresses. **D** – Cross-section of infested trees reveals a complex network of tunnels or brood galleries, formed by the female beetles as they establish reproductive activity. **E** – When closely inspecting the galleries of infested and damaged plant tissue, it is possible to discover the beetles at their different life stages (adult beetles as well as larvae and eggs). Images obtained from Eskalen *et al.* (2012).

b. The fungal symbionts of the PSHB and the causal pathogen of FD

When considering the pathogenic organism responsible for the causation of FD, it is important to note that the PSHB and many other ambrosia beetles have been shown to consistently exhibit some form of symbiosis with more than one fungal partner (Lynch *et al.*, 2016). It is usually the case, however, that one of the fungal species is classified as the primary symbiont. In the case of FD, the beetle has been associated with three re-occurring fungal partners: *Paracremonium pembeum* sp. nov., *Graphium euwallaceae* sp. nov. and *F. euwallaceae* (Lynch *et al.*, 2016, Paap *et al.*, 2018). Preliminary studies have hypothesised that *P. pembeum* and *G. euwallaceae* are not considered to be pathogenic organisms, instead, evidence suggests that these fungal partners vary incrementally in their importance to the beetle, by providing specialised nutrition to the different life stages of the beetle and its larvae (Freeman *et al.*, 2016). Conversely, the last-mentioned fungus – *F. euwallaceae* – has been repeatedly described as the causal agent for the disease caused by the PSHB pest complex (Eskalen *et al.*, 2012, Freeman *et al.*, 2016, García-Avila *et al.*, 2016, van den Berg *et al.*, 2019), but details regarding its interaction with the other symbionts are still gravely understudied.

The ascomycete genus of *Fusarium* is an abundant group of filamentous fungi, classified within the Hypocreales order (Chelkowski, 2014). Most of these *Fusaria* occur as harmless saprobes and active members within most soil microbial communities. This group of fungi is also conversely one of the fungal genera that is home to some of the most destructive plant pathogens (Moore *et al.*, 2001). Accurately differentiating between these organisms and establishing taxonomy based solely on

morphological characteristics has been largely impractical (DeSalle *et al.*, 2005). However, with the advent of DNA sequencing and phylogenetic inferencing, these once cryptic and indistinguishable organisms have started to enjoy more detailed and concise classifications, based on levels of DNA similarity or difference between these related organisms (Tautz *et al.*, 2003).

The primary, pathogenic, ambrosial fungus, regarded as the causal agent of FD, has been classified as *F. euwallaceae* (Eskalen *et al*., 2012). When cultured on ½ PDA (Potato dextrose agar), some key, diagnostic, morphological characteristics can be used to distinguish *F. euwallaceae* from some other environmentally occurring micro-organisms. However, the use of morphology alone cannot be used in isolation to identify *F. euwallaceae,* as false-positive identification may occur due to morphological overlap with many closely related species. These characteristics include a mycelial culture that grows radially outward, above solid agar medium and phenotypically presents as whitish-yellow, eventually darkening in colour as the culture ages. Significantly aged cultures are sometimes observed as becoming dark purple to grey. When cultured and incubated in the dark, at a constant temperature of 25°C, the mycelia takes about 10 - 15 days to completely colonise the surface of a 90 mm ½ PDA nutrient agar plate.

The macro-conidia of *F. euwallaceae* are generally relatively wide, straight, stout and robust, with the apical cell morphology being curved, rounded and blunt. The macro-conidia are usually 3- to 7-septate and are abundant in sporodochia. Oval or reniform micro-conidia are formed in round, false heads and are abundant in the aerial mycelia. Chlamydospores are often produced abundantly, in pairs and may be intercalary in

the hyphae or formed terminally on short, lateral branches. The variety of mycelial structures and spores vary widely depending on the environment, with the morphology and production of different structures being highly-sensitive to surrounding potassium ion (K^+) levels. Microscopic descriptions of mycelia and spores obtained from the Fusarium Laboratory Manual (Leslie & Summerell, 2008). The culture morphology and microscopic appearance of *F. euwallaceae* are shown in **Figure 3**.

Due to the fact that knowledge and research-based on this specific pathogen are very limited, the precise lifecycle and behavioural ecology, particularly its interaction with the PSHB, still remain largely unknown. Specific features of the lifecycle can however broadly be inferred from the species complex that it is part of. In general, most members of the FSSC are considered necrotrophic plant pathogens, meaning that the mode of action is predominantly defined by rapid colonisation and rapid host cell death (Laluk & Mengiste, 2010). Necrotrophic pathogens do not produce intracellular feeding structures such as haustoria, instead, they progress at localised sites, where they cause vast cellular damage at both intra- and intercellular levels. These pathogens therefore rely on the secretion of lytic enzymes and toxins, such as cellulases, to break down the host's defences and tissues (Alfano & Collmer, 1996), in order to obtain maximum nutritional benefit in the shortest time possible.

Most *Fusarium solani* spp. are globally distributed and are abundantly found in native soils as both non-pathogenic and highly pathogenic members of the microbial communities, however, *F. euwallaceae* has yet to be isolated from areas that are free of the presence of its vector organism, in the directly surrounding area (Freeman *et al.,* 2016).

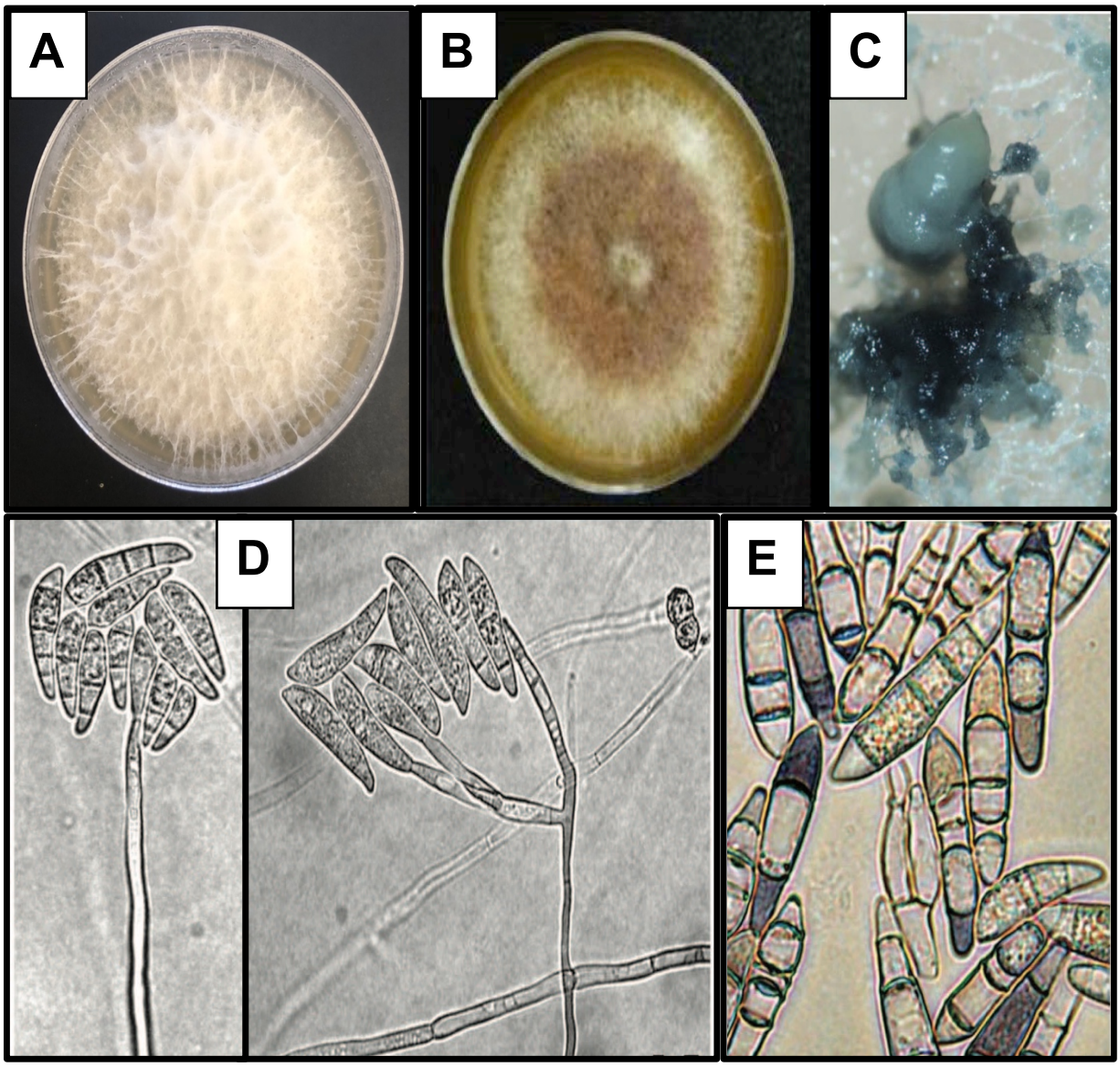


Figure 3: The culture and microscopic morphology of *Fusarium euwallaceae*. **A** – *F. euwallaceae* cultured on Potato Dextrose agar (PDA), 7 days old, can be observed as fuzzy and soft white mycelia. **B** - *F. euwallaceae* cultured on PDA, approximately 30 days old, observed as a more dense, dark purple/grey mycelia. **C** – Greyish conidial masses or clumps are formed when cultured on PDA. **D** – Both images indicate microscopic photos of hyphae with long-stalked, aerial conidiophores and attached conidia. **E** – A microscopic photo of the large, septate macro-conidia, which are formed atop the conidiophores. Images obtained and modified from Laluk & Mengiste (2010), Freeman *et al.* (2013) and Lynch *et al.* (2016).

Part of the difficulty in identifying *Fusaria* at a species level is that the genus contains many different species. Oftentimes, these closely related organisms are virtually indistinguishable when comparing their phenotypes and morphologies (Babadoost, 2018). For this reason, it has become conventional to study the DNA sequences of these organisms, alongside their morphological characteristics (van Diepeningen & de Hoog, 2016). This is done by studying DNA "barcodes" in the form of housekeeping genes (HKGs) or highly-conserved gene regions, particularly with reference to the degree of difference between the individual nucleotides among related species. This approach of considering the DNA of an organism, in tandem with its appearance, not only helps to distinguish them taxonomically but it also helps to speed up the diagnosis of disease, through accurate and measurable comparisons. These comparisons, with regards to DNA sequences, allow for the further clarification of species identities and degrees of relatedness (Kress *et al.*, 2015). Various gene sequences may also be used and combined, as barcode sequences depending on the nature of the study.

An additional tool that can be combined with the use of morphological and DNA comparisons, to identify unknown organisms, is considering the phylogenetic background of the organism. The phylogeny of an unknown organism, of a suspected genus, can accurately reveal the unknown organism's species identity, as well as levels of similarity or difference between members of the same species (Guadet *et al.*, 1989), when a particular gene is considered. This is achieved by superimposing an obtained DNA sequence, of an unknown organism, into a phylogenetic structure the same DNA barcode of known organisms. Following the generation of a maximum-likelihood phylogenetic tree, the exact positioning of the unknown DNA sequence can further be used as a means to clarify the identity of an organism (O'Donnell, 1996).

From a taxonomic standpoint, *Fusaria* that maintain mutualistic relationships with any ambrosia beetles are monophyletically grouped within the Ambrosial *Fusarium* Clade (AFC) (O'Donnell *et al.*, 2015). Phylogenetic studies regarding these ambrosial *Fusaria* have resolved the AFC within the FSSC, using a multi-locus phylogenetic approach. The gene regions involved in this study and most studies involving the taxonomic deciphering of the *Fusarium* genus include the internal transcribed spacer (ITS) together with the translation elongation factor-1 alpha (*TEF-1α),* β-tubulin, the DNA-directed RNA polymerase II subunit 1 (*RPB1*) and the DNA-directed RNA polymerase subunit 2 (*RPB2)* (O'Donnell *et al.*, 2015). This approach facilitates the description of new species within the AFC, but it also allows pathologists to determine similarities or differences in haplotypes, compared to already described species (Kasson *et al.*, 2013).

c. The FD insect vector – the polyphagous shot hole borer ambrosia beetle

In a similar way to the pathogen that is harboured by this insect-vector, the means of identification for diagnosis of the PSHB has also proven problematic. The ambrosia beetle *E. fornicatus* Eichhoff. *sensu lato* is a species complex that is comprised of three morphologically indistinguishable members (Gomez *et al.*, 2018). These include the Polyphagous Shot Hole Borer (PSHB), the Tea Shot Hole Borer (TSHB) and the Kuroshio Shot Hole Borer (KSHB). Recently, however, studies have re-evaluated and further reassessed the cytochrome oxidase c subunit I (*COX1*) barcoding gene region to assign specimens to separate species and clades (Gomez *et al.*, 2018, Smith *et al.*, 2019). The current taxonomic structure is as follows – the PSHB is revised to *Euwallacea fornicatus* Eichhoff., the TSHB clade a is revised to *Euwallacea perbrevis*

Eichhoff. and clade b is revised to *Euwallacea fornicatior* Eggers. The KSHB is revised to *Euwallacea kuroshio* Gomez and Hulcr.

This provides a step towards evidence-based species delineation, necessary for addressing the overlap in morphological characters of these related pest species. The most suitable way to identify and detect these beetles, from a pathology standpoint, is therefore based on a similar approach, as used for the pathogen that it harbours. These factors include considering key morphological characteristics, the DNA sequence of a conserved gene region (*COX1*) (Cooperband *et al.*, 2016) as well as the phylogenetic structure that is observed.

Due to large number of beetle species in most environments, a practical starting point is to consider the signature morphological characteristics of the PSHB. Some of the diagnostic characteristics that are considered are the appearances of the head shape and size, pronotum width and length, antennae, conspicuous features such as serrations or hairs on the head and elytra, punctures on the lateral margin, leg shape and size, elytral length and width and degree of elytral declivity (Stouthamer *et al.*, 2017). One key characteristic that can distinguish non-ambrosia beetles and other ambrosia beetles from the PSHB, is the fact that the PSHB has no conspicuous surface features, except for hairs that are arranged in rows across the entire elytron, but is completely free of any serrations at the elytral declivity (Vega & Hofstetter, 2014). The surface of the declivity is unarmed by any large projections, only covered with strial punctures and interstrial tubercles, granules and hairs (Wood, 1982). The PSHB is commonly darkly-coloured (usually brown or black) and has a smooth, broadened pronotum – a prominent plate-like structure that covers part of the head

and thorax (Paap *et al.*, 2018). The colour of the beetle remains uniform throughout its body with variable sizes from 2 mm to 4.5 mm. The anterior margin of the pronotum is also smooth and devoid of serrations. The PSHB has a gently descending elytron that is never drastically sloped, indented or truncated (Bateman & Hulcr, 2014). The elytron is about 1.5x longer than the head and pronotum (Kirkendall *et al.*, 2015). Some of these characteristics can be observed from the planar angles and are illustrated in **Figure 4**.

It is also important to observe the lifecycle (**Figure 5**) and key aspects of this vector. It is essential to holistically approach Fusarium Dieback and its various components, as some of the aspects of its behavioural ecology are unique. Considering the areas of interaction during the vector's lifecycle allows for a better understanding of the overall disease and during which phases of the disease cycle it is feasible to attempt to apply an appropriate control measure.

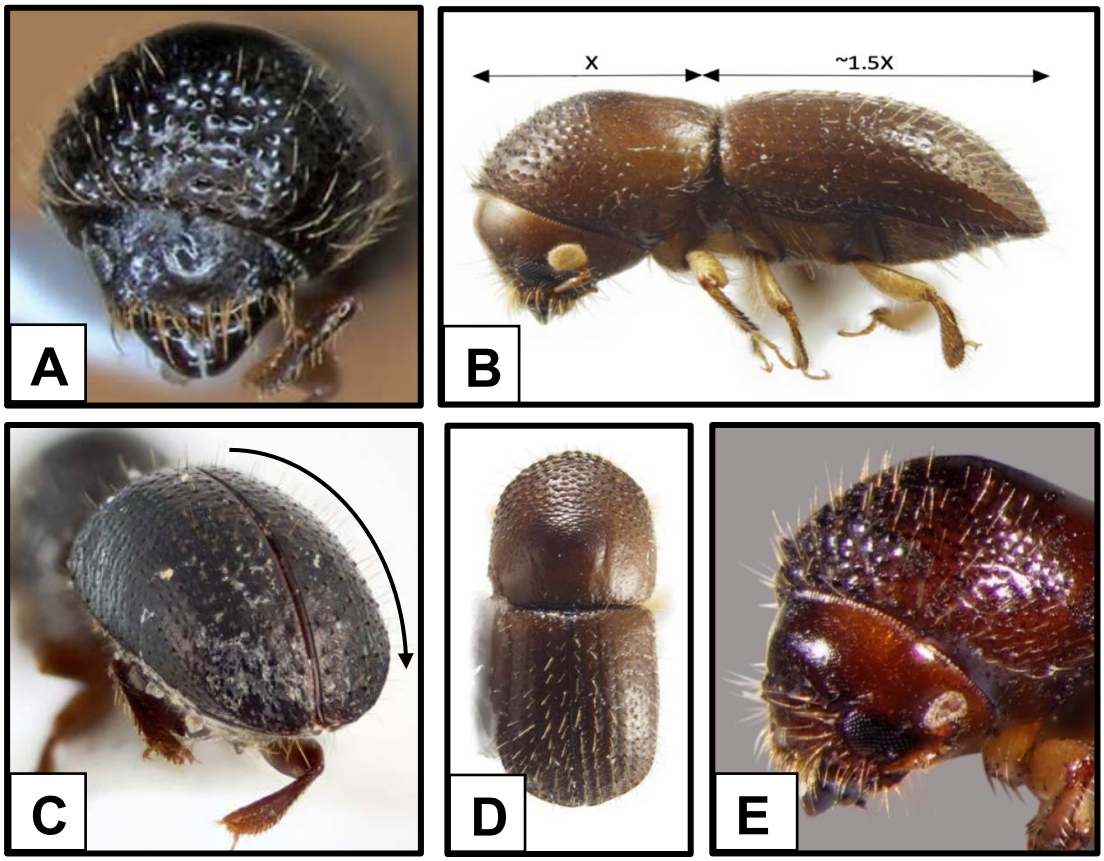


Figure 4: Microscopic images of the polyphagous shot hole borer - PSHB (*Euwallacea fornicatus).* **A –** The anterior, cranial view of the PSHB. **B –** The lateral view of the PSHB. This planar angle shows the ratio between the size of the pronotum and head relative to the size of the elytron. The entire beetle's body ranges from 2 mm to 4.5 mm, with the elytron being approximately 1.5x as long as the head and pronotum. **C –** The posterior-oblique, caudal view of the PSHB. This planar angle indicates how the elytron slopes gently downwards (indicated with the arrow) and how the elytron is not jagged or truncated. **D –** The dorsal view of the PSHB. This planar angle shows how the body of the PSHB is free of any surface features, except for the hairs that are arranged in rows across the length of the elytron**. E –** The anterior, cephalic view of the PSHB. This planar angle, along with **image A**, indicates the appearance of the antennae and the antennal club, as well as the presence of some hairs and bumps on the external surface of the pronotum. The pre-oral mycangium is not shown here. Images obtained Bateman & Hulcr (2014).

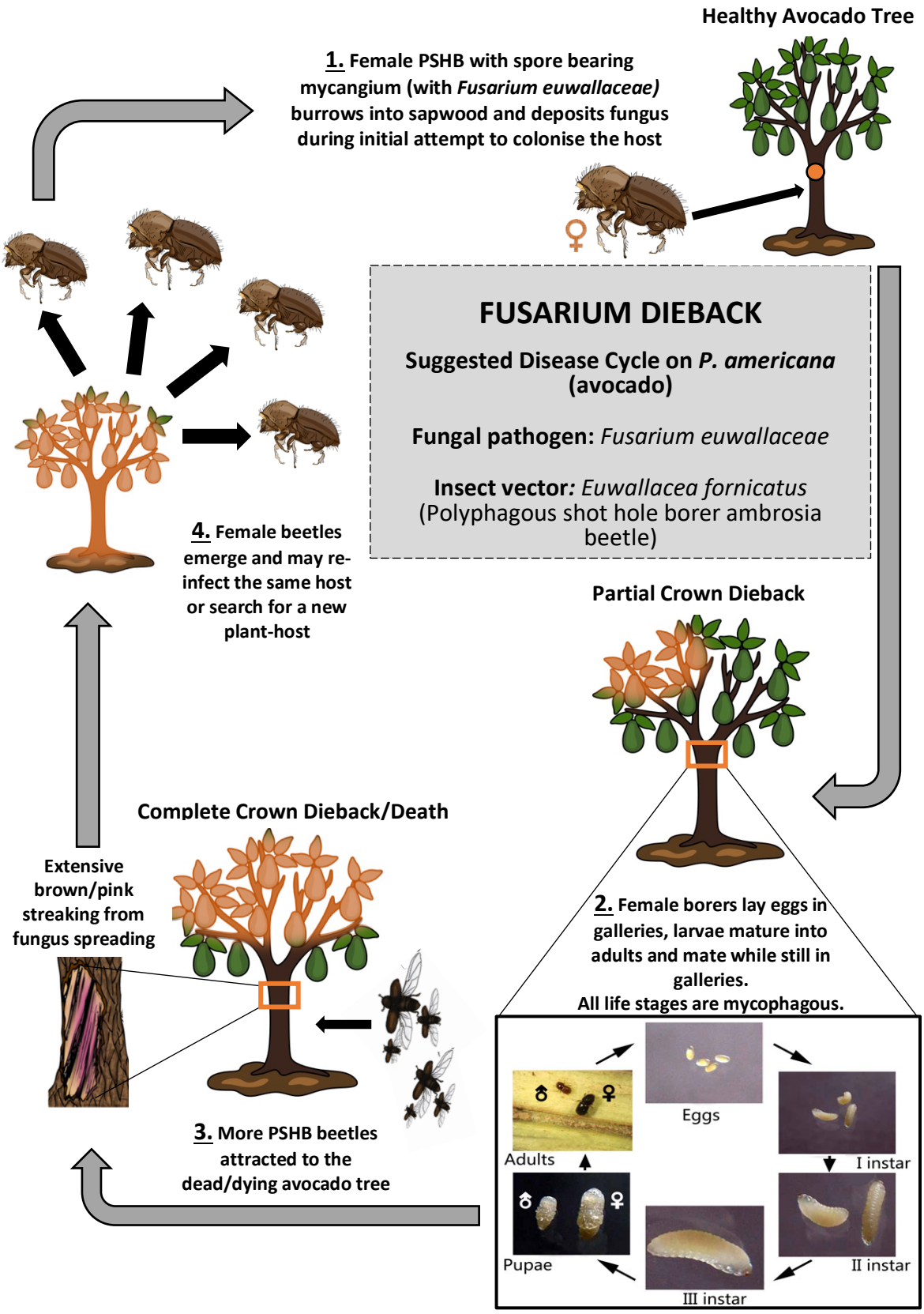


Figure 5: The lifecycle of the polyphagous shot hole borer - PSHB (*Euwallacea fornicatus*). The lifecycle shows the interaction between the vector and the pathogen it carries (*Fusarium euwallaceae)*, causing Fusarium Dieback on avocado.

CONTROL AND MANAGEMENT STRATEGIES FOR FUSARIUM DIEBACK

Due to the fact that this disease is harbored and distributed by means of an insect vector, the control strategies need to be cautiously and holistically considered. The efficacy and efficiency of control means must be aimed at long-term prophylaxis and must be applicable in real-world scenarios. The cost of implementation is also of particular significance, because attempting to control an organism that can freely traverse most land borders and geographical barriers, oftentimes becomes a very large or impractical financial undertaking (Rugman-Jones & Stouthamer, 2016). Along with these factors, perhaps the most important consideration is the impact of applying control strategies on the environment. Attempting to control this insect pest by means of conventional approaches is largely ineffective due to the fact that most of the beetle's life stages are spent within the host tissue and attempting to control the emerging females (the males are flightless) is unrealistic. For these reasons, several tailored approaches have been proposed.

a. Control of the insect-vector – *Euwallacea fornicatus*

The control of this pest complex can be broadly divided into two divergent approaches. The first being to control the insect-vector and the other being to control the pathogenic micro-organism responsible for disease. The main and currently most effective means of management, to prevent the spread of this beetle, is to apply early detection techniques to identify areas of infestation, to remove the sources/reservoirs of inoculation and to destroy the infested woody plant material (Hulcr & Stelinski, 2017). Single pieces of wood (from as low as 500 grams) have been shown to be capable of

housing hundreds of beetles, so individual sources of infested wood, that can be spread unknowingly, may result in countrywide dispersal. In areas where this pest has established, pruning and incineration of the infested plants have been recommended as effective cultural practices. Treatment of infested wood via incineration has obvious shortcomings, therefore the development of an alternative option is of high priority. Studies have shown that fine-scale wood chipping, of sources of infested plant material and prolonged exposure to sunlight in the form of solarisation, can be a more economical and sustainable solution (Jones & Paine, 2015). However, these beetles are very small in size, so ineffective or irresponsible chipping and dispersal of the chipped wood, containing potentially live beetles, may result in the further dispersal of the pest.

Chemical control, in the form of insecticides and pesticides (Jones & Paine, 2018) such as the use of emamectin benzoate or bifenthrin have been considered. However, applying spray insecticides to control this pest oftentimes results in negative implications, by affecting the beneficial insect species within an environment. Further, the action of many insecticides relies on being injected directly into the trunk of a plant and depends on the action of the beetle feeding directly from the woody material. Since this mycophagous ambrosia beetle does not feed directly from the host tissue (Hulcr & Stelinski, 2017), in any of its life stages, many of these insecticides are deemed ineffective by default. Along with this factor, it is often unfeasible to inject insecticides into the tissue of fruit trees, as it may compromise the organic status of the resultant plant and fruit.

Another means of control, that can be applied to limit the spread of the beetles, is to populate a commercially active area, such as an orchard, with chemical lures/traps (Carrillo *et al.*, 2015). The intention is to fill chemical lures with compounds, such as ethanol or sex pheromones (pherolures) and to hang the lures from branches, in areas where PSHB presence is suspected. Ethanol has proven ineffective against successfully luring the PSHB, therefore, these traps may include other volatile compounds such as quercivorol, α-copaene or verbenone (Dodge *et al.*, 2017) which have been shown to attract and then subsequently to retain the beetles in a one-way funnel system. This can serve as a tool to limit their spread by monitoring and detecting the presence of the PSHB in an area. Similarly, these traps may also be filled with deterrents, such as piperitone, which aims to repel the beetle from an area (Owens *et al.*, 2018). The fundamental flaw of this approach has two sides, the first being the long-term sustainability. These compounds have been shown to retain efficacy for very short time-periods only, unless the traps are individually refilled on a regular basis, presenting severe monetary constraints (Jones *et al.*, 2017). The other problem with luring as a control means is that the area of activity is often insufficient and may only have an effect on a very limited area (Dodge *et al.*, 2017). These traps have also proven to be largely ineffective in areas with high wind speeds.

b. Control of the ambrosial pathogen – *Fusarium euwallaceae*

In contrast to trying to control the vector, several studies have been done to evaluate the effect of controlling the pathogen itself, as it comes into contact with the host. The most obvious means of control is by applying chemical control, in the form of broad-spectrum triazole fungicides such as propiconazole, tebuconazole, and metconazole

(Grosman *et al.*, 2019), to infested trees. In the same way as applying chemicals to control the vector, similar limitations are true for the pathogen. Spray-fungicides applied to infested plants are often only effective at sterilising the outer surface of the plant tissue and as such the pathogenic inoculum, deep within the plant tissue remains unaffected (Jones *et al.*, 2017). As is the case with trunk injections, aimed at controlling the beetle, fungicides which are directly injected into the tissue of fruit trees may ultimately compromise the value and organic status of the resultant fruit, as well stressing the tree by creating a wound at the site of injection (Flower *et al.*, 2015).

In the quest to provide a sustainable solution as a control means, the possibility of applying an organism as a biological control means, against the pathogen has also been evaluated. The gram-positive bacterium - *Bacillus subtilis,* was successfully isolated from healthy avocado roots and has been shown to have promising application as a biological control organism (Guevara-Avendaño *et al.*, 2018). *B. subtilis* has been closely linked with the production of secondary metabolites and compounds that act in antagonism to *F. euwallaceae*. These compounds generally consist of antifungal lipopeptides such as surfactin, fengycin, and iturin A (Jones *et al.*, 2017). *B. subtilis* as well as some *Trichoderma* spp*. (*Benítez *et al.,* 2004*)* have shown high potentials as control means as they are directly able to inhibit and limit the growth of many fungal pathogens, to a significant degree, when applied at the appropriate point during infection or to the soil. This approach may aid in the reduction of the impact of synthetic chemistries and may further aid in avoiding fungicide-resistant pathogen populations. The application (through spray or trunk injections) of *B. subtilis* is unfortunately comparable to the application of fungicides, with regards to the

duration of effectiveness (Muis, 2016). Like fungicides, the use of biocontrol agents has been shown to confer only short-term protection against the PSHB pest complex.

Extremely high value, individual trees may be considered as potential candidates for repeated/sustained biocontrol treatment (*B. subtilis* foam spray), such as trees that represent national heritages/societally impactful trees. For example, some South African trees have been deemed “champion trees” for surviving for hundreds of years and their presences have become a symbol of native culture and/or sources of tourism, such as the Outeniqua yellowwood (*Podocarpus falcatus*) in Knysna, in the Western Cape, which is nearly 900 years old and stands 40m tall or the English oak tree (*Quercus robur*) in Sophiatown, in Johannesburg, in Gauteng. This tree is estimated to be over a century old and is considered a natural relic and landmark (Stapleton, 1995).

Another way of sustainably responding to the threat posed by the PSHB and its fungal symbionts is to consider the development or breeding of pathogen-resistant plants. This process may involve screening cultivars or plant varieties for resistance to specific pathogens and then attempting to breed and grow pathogen-resistant or pathogen-tolerant varieties (Huet, 2014). The acquisition of pathogen-resistant plants may also be done by developing transgenic plants, through transformation, by inserting gene sequences, which could confer resistance (Collinge, 2016). The resistance, in this case, stems from the ability of the plant to synthesise and produce compounds from an inserted gene sequence, encoding for the desired resistance compound (Collinge, 2016). Both approaches can be used to increase resistance to pathogens or alternatively, to confer higher resistance to herbicides, pesticides or fungicides, which

will allow for a greater level of control or interference by human activity. However, the general trend is that breeding/development of these pathogen-resistant plants results in a cost to the productivity or quality of produced plant products i.e. fruit or timber, making it less feasible in many applications (Bidney *et al.*, 2000). Unfortunately, this approach, as a means to answer the threat from the PSHB, does not confer any protection to already growing and currently threatened trees.

For all these reasons and individual limitations, the most likely solution to control and limit the spread the PSHB is an integrated approach, that makes use of timely detection and a combination of the aforementioned control techniques, to control the beetle as well as its fungal symbiont.

THE PSHB IN SOUTH AFRICA: DISTRIBUTION AND BARRIERS TO DETECTION

Due to the international notoriety of this highly destructive pest complex and the threat of other pests to local ecosystems, sentinel tree plantings are conducted as a means to record new and emerging host-pest interactions within an area. In 2018, one such a survey, performed in the KwaZulu-Natal National Botanical Gardens, revealed the presence of a beetle and its fungal symbiont, damaging London Plane trees (*Platanus x acerifolia)* (Paap *et al.*, 2018). Further studies verified the identity of the damaging pathogen-vector as being part of the *E. fornicatus senu lato* species complex, in particular, a 100% match to the H33, internationally invasive, haplotype. In addition, the primary fungal symbiont – *F. euwallaceae* was isolated from the diseased plant tissue (Paap *et al.*, 2018).

It is important to consider the potential hazard that such an emerging threat can pose. The local avocado industry in South Africa spans across a large variety of areas and provinces and commercially encompasses about 17 500 hectares of arable land, with a permanent workforce of about 2.5 workers/hectare (South African Avocado Growers Association, 2019). From a South African economic standpoint, this means that avocadoes are involved in subsistence agriculture to be consumed locally and a further $64.1 million (or approximately R 940 000 000) is generated, in exportation-only value (Hass Avocado Board, 2019). This alludes to the potential damage that such a pest complex could impart if cost-effective and conscientious solutions are not in place. More recently, the first report of the PSHB and its fungal symbiont damaging *P. americana* (avocado), in Johannesburg, Gauteng, has been published (van den Berg *et al.*, 2019). This suggests that the pest complex has continued to spread rapidly through South Africa. The list of hosts affected since is publicly available on the FABI (Forestry and Agriculture Biotechnology Institute) website, at https://www.fabinet.up.ac.za/pshb and are illustrated in **Table 1 and 2.**

Table 1: The exotic- and native, reproductive host plants that have been observed to be affected in South Africa, since the it was first detected causing damage to London Plane trees (*Platanus x acerifolia*) (Paap *et al*., 2018).

REPRODUCTIVE HOST TREES IN SA			
Exotic species		**Native SA species**	
Latin name	**Common name**	**Latin name**	**Common name**
Acacia melanoxylon	Blackwood	*Combretum krausii*	Forest Bushwillow
Acacia mearnsii	Black Wattle	*Erythrina caffra*	Coast Coral Tree
Acer buergerianum	Trident (Chinese) Maple	*Podalyria calyptrata*	Water Blossom Pea
Acer negundo	Boxelder	*Psoralea pinata*	Fountain Bush
Acer palmatum	Japanese Maple	*Salix mucronata*	Cape Willow
Brachychiton discolor	Pink Flame Tree		
Gleditsia triacanthos	Honey Locust		
Liquidambar styraciflua	American Sweetgum		
Magnolia grandiflora	Southern Magnolia		
Persea americana	Avocado		
Platanus x *acerifolia*	London Plane		
Quercus palustris	Pin Oak		
Quercus robur	English Oak		
Ricinus communis	Castor Bean		
Salix alba	White Willow		

Table 2: The exotic- and native, non-reproductive host plants that have been observed to be affected in South Africa, since the it was first detected causing damage to London Plane trees (*Platanus x acerifolia*) (Paap *et al*., 2018).

NON-REPRODUCTIVE HOST TREES IN SA			
Exotic species		**Native SA species**	
Latin name	**Common name**	**Latin name**	**Common name**
Bauhinia purpurea	Butterfly orchid tree	*Bauhinia galpinii*	Pride Of de Kaap
Betula pendula	Silver birch	*Buddleja saligna*	False Olive
Camellia japonica	Common camellia	*Calodendrum capense*	Cape Chestnut
Carya illinoinensis	Pecan nut	*Calpurnia aurea*	Geelkeurboom
Ceiba pentandra	Kapok	*Combretum erythrophyllum*	River Bushwillow
Cinnamomum camphora	Camphor	*Cordia caffra*	Septee Tree
Citrus limon	Lemon	*Cussonia spicata*	Cabbage Tree/ Kiepersol
Citrus sinensis	Orange	*Diospyros dichrophylla*	Star Apple
Eriobotrya japonicum	Loquat	*Diospyros lycidioides*	Monkey Plum
Erythrina livingstoniana	Aloe coral tree	*Ekebergia capensis*	Cape Ash
Eucalyptus camaldulensis	River red gum	*Erythrina lysistemon*	Common Coral Tree
Ficus carica	Common fig	*Ficus natalensis*	Natal Fig
Fraxinus excelsior	European ash	*Grewia occidentalis*	Cross Berry
Jacaranda mimosifolia	Jacaranda	*Gymnosporia buxifolia*	Spike Thorn
Melia azedarach	Syringa	*Halleria lucida*	Tree Fuchsia
Morus sp.	Mulberry	*Harpephyllum caffrum*	Wild Plum
Platanus occidentalis	American plane	*Melianthus major*	Honey Flower
Platanus racemosa	Californian plane	*Nuxia floribunda*	Forest Elder
Plumeria rubra	Frangipani	*Olea europea* subsp. *africana*	Wild Olive
Populus nigra	Lombardy poplar	*Podocarpus falcatus*	Outeniqua Yellowwood
Prunus nigra	Black plum	*Podocarpus henkelii*	Henkel's Yellowwood

Accurate and unambiguous species descriptions are the fundamental foundations for detecting organisms that are linked to disease causation or disease interaction. A re-occurring boundary to the diagnosis or treatment barrier of the PSHB is due to imprecise descriptions or taxonomy regarding the causal agent for this disease. Therefore, it is crucial to establish correct and accurate species identities, through thoroughly structured reference frameworks of type-specimens. The PSHB and its closely related organisms, as well as the pathogen that it harbors have only recently started being correctly described, so the deficiency of pre-existing knowledge imparts major difficulties on early detection.

In addition to facing difficulties with the taxonomy of the pest complex, it is common to find many insects, particularly beetle species associated with burrowing into stressed plants, as evolution has allowed for the diverse speciation of beetles (Bracewell *et al.*, 2018). It is therefore sometimes overwhelming to attempt to identify and detect the responsible vector-organism within the background generated by non-invasive, albeit very similar looking, beetle species. Together with this factor, instances of FD have been shown to present extreme variations on the symptoms caused, from host to host (Eskalen *et al.*, 2013), which further hampers the process of early detection. General practices to identify insects are therefore not always practical as specimens suspected to be the PSHB need to be further studied and their identities need to be confirmed, through DNA practices, resulting in the loss of the specimen. Along with this, these beetles possess exoskeletons (Brockerhoff *et al.*, 2006) with extremely robust and hardened features, meaning that conventional DNA extraction protocols, reliant on fresh and well-preserved specimens, regularly result in inconclusive results and a loss of the specimen.

Initial detection of the symptoms caused by the PSHB also requires an individual who is well versed with the symptoms for the disease, as there are many factors that could lead to false-positive identifications (such as holes caused by non-ambrosia beetles, varying field-symptoms, woodpecker holes or trunk injections). In the light hereof, the primary source for the potential detection of new and emerging diseases places a heavy reliance on establishing a well-informed public to monitor the spread of this disease (Holmes, 2008). There is often a lack of knowledge and awareness and a long lag period, that can accelerate the threat and spread of an emerging disease.

CONCLUSION

Existing knowledge, regarding this beetle-fungus pest, suggests that the threat posed to local industries is of importance. In areas where these ambrosia beetles have caused damage, impacts on both biodiversity and the economy follow (Ploetz *et al.*, 2013). Biodiversity is drastically impacted through the killing of native/indigenous species by these pest complexes. Environments that have suffered losses to their native tree populations, as a result of beetle-fungus damage, may become occupied by alien or invasive tree species (Bentz *et al.*, 2010). This form of environmental damage often occurs in areas that are not routinely monitored, so the loss is mostly unapparent, however the long-term effect warps and alters the biodiversity structure of an environment. Unapparent losses, such as these, are only a component of the actual impact that these pest complexes cause.

As far as an economic perspective is concerned, the beetle-fungus pest complex has far-reaching effects. In addition to being able to damage plant tissue, reducing the resale value of timber, agriculture may also be significantly affected (Ranger *et al.*,

2015). In Israel and the United States of America, where avocado is grown as a major fruit crop, the yield loss due to this pest has been substantial. In a country such as South Africa, the magnitude of the potential loss is concerning.

Unlike many other fungal bearing beetles, the polyphagous shot hole borer is also, as the name implies, not a specialist pest. Rather, the PSHB is considered to be a generalist pest, as such it threatens a large variety of reproductive and non-reproductive plant species, from as many as 58 different plant families (Eskalen *et al.*, 2013). This is noteworthy, as the threat that is posed, by this pest, affects not only the agricultural industry but also the biosecurity of large scale plantations and naturally occurring forests, as well as urban and ornamental plant varieties. The consequence of this, is that continued research on this topic is imperative, towards understanding the basic aspects of the disease lifestyle, as well as determining the geographical spread of this pest, in South Africa and to assess the potential threat to the local avocado growing industry.

REFERENCES

Alfano JR & Collmer A (1996) Bacterial pathogens in plants: Life up against the wall. *The Plant Cell* **8**: 1683.

Altieri M & Nicholls C (2004) *Biodiversity and pest management in agroecosystems*. CRC Press.

Aylward FO, Currie CR & Suen G (2012) The evolutionary innovation of nutritional symbioses in leaf-cutter ants. *Insects* **3**: 41-61.

Babadoost M (2018) Fusarium: Historical and Continued Importance. *Fusarium-Plant Diseases, Pathogen Diversity, Genetic Diversity, Resistance and Molecular Markers* IntechOpen.

Bateman C & Hulcr J (2014) A guide to Florida's common bark and ambrosia beetles. *University of Florida IFAS Extension* 1-36.

Beaver RA, Wilding N, Collins N, Hammond P & Webber J (1989) Insect-fungus relationships in the bark and ambrosia beetles. *Insect-fungus Interactions* 121-143.

Benítez T, Rincón AM, Limón MC & Codon AC (2004) Biocontrol mechanisms of *Trichoderma* strains. *International Microbiology* **7**: 249-260.

Bentz BJ, Régnière J, Fettig CJ, Hansen EM, Hayes JL, Hicke JA, Kelsey RG, Negrón JF & Seybold SJ (2010) Climate change and bark beetles of the western United States and Canada: Direct and Indirect effects. *BioScience* **60**: 602-613.

Bidney DL, Scelonge CJ, Charne DG, Mancl MK, Falak I, Nazarian KA & Cole GS (2000) Production of pathogen resistant plants. Washington, DC: U.S. Patent and Trademark Office.

Biedermann PH, Klepzig KD & Taborsky M (2009) Fungus cultivation by ambrosia beetles: behavior and laboratory breeding success in three xyleborine species. *Environmental Entomology* **38**: 1096-1105.

Borden JH (1988) The striped ambrosia beetle. *Dynamics of forest insect populations* 579-596. Springer.

Bracewell R, van der pool D, Good J & Six D (2018) Cascading speciation among mutualists and antagonists in a tree–beetle–fungi interaction. *Proceedings of the Royal Society B: Biological Sciences* **285**: 20180694.

Bright C (1999) Invasive species: pathogens of globalization. *Foreign Policy* **50**: 6-15.

Brockerhoff EG, Bain J, Kimberley M & Knížek M (2006) Interception frequency of exotic bark and ambrosia beetles (Coleoptera: Scolytinae) and relationship with establishment in New Zealand and worldwide. *Canadian Journal of Forest Research* **36**: 289-298.

Byers JA & Maoz Y (2017) Attraction of the *Euwallacea* sp. near *fornicatus* (Coleoptera: Curculionidae) to quercivorol and to infestations in avocado. *Journal of Economic Entomology* **110**: 1512-1517.

Carrillo D, Duncan RE & Peña JE (2012) Ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) that breed in avocado wood in Florida. *Florida Entomologist* 573-579.

Carrillo D, Narvaez T, Cossé AA, Stouthamer R & Cooperband M (2015) Attraction of *Euwallacea* nr*. fornicatus* (Coleoptera: Curculionidae: Scolytinae) to lures containing quercivorol. *Florida Entomologist* **98**: 780-783.

Carrillo D, Cruz L, Kendra P, Narvaez T, Montgomery W, Monterroso A, De Grave C & Cooperband M (2016) Distribution, pest status and fungal associates of *Euwallacea* nr. *fornicatus* in Florida avocado groves. *Insects* **7**: 55.

Carter W (1962) Insects in relation to plant disease. *Insects in Relation to Plant Disease*. Interscience Publishers.

Chelkowski J (2014) *Fusarium: Mycotoxins, Taxonomy, Pathogenicity*. Elsevier.

Cleaveland S, Haydon D & Taylor L (2007) Overviews of pathogen emergence: Which pathogens emerge, when and why? *Wildlife and Emerging Zoonotic Diseases: The Biology, Circumstances and Consequences of Cross-Species Transmission* 85-111. Springer.

Collinge DB (2016) *Plant pathogen resistance biotechnology*. Wiley Online Library.

Cooperband MF, Stouthamer R, Carrillo D, Eskalen A, Thibault T, Cossé AA, Castrillo LA, van den berg JD & Rugman-Jones PF (2016) Biology of two members of the *Euwallacea fornicatus* species complex (Coleoptera: Curculionidae: Scolytinae), recently invasive in the USA, reared on an ambrosia beetle artificial diet. *Agricultural and Forest Entomology* **18**: 223-237.

Coyle DR, Booth DC & Wallace MS (2005) Ambrosia beetle (Coleoptera: Scolytidae) species, flight, and attack on living eastern cottonwood trees. *Journal of Economic Entomology* **98**: 2049-2057.

Crowl TA, Crist TO, Parmenter RR, Belovsky G & Lugo AE (2008) The spread of invasive species and infectious disease as drivers of ecosystem change. *Frontiers in Ecology and the Environment* **6**: 238-246.

Crowson RA (2013) *The biology of the Coleoptera*. Academic Press.

Currie CR, Mueller UG & Malloch D (1999) The agricultural pathology of ant fungus gardens. *Proceedings of the National Academy of Sciences* **96**: 7998-8002.

Dávila JA, Rosenberg M, Castro E & Cardona CA (2017) A model biorefinery for avocado (*Persea americana* Mill.) processing. *Bioresource Technology* **243**: 17-29.

DeSalle R, Egan MG & Siddall M (2005) The unholy trinity: taxonomy, species delimitation and DNA barcoding. *Philosophical Transactions of the Royal Society B: Biological Sciences* **360**: 1905-1916.

Dobson A, Barker K & Taylor SL (2013) *Biosecurity: the socio-politics of invasive species and infectious diseases*. Routledge.

Dodge C, Coolidge J, Cooperband M, Cossé A, Carrillo D & Stouthamer R (2017) Quercivorol as a lure for the polyphagous and Kuroshio shot hole borers, *Euwallacea* spp. nr. *fornicatus* (Coleoptera: Scolytinae), vectors of Fusarium dieback. *PeerJ* **5**: e3656.

Dolinko A, Carmarán C, Ceriani-Nakamurakare E, Slodowicz M & Gonzalez-Audino P (2016) Mycobiota associated with the ambrosia beetle *Megaplatypus mutatus*: threat to poplar plantations. *Forestry: An International Journal of Forest Research* **89**: 191-200.

Eskalen A, Gonzalez A, Wang D, Twizeyimana M, Mayorquin J & Lynch S (2012) First report of a *Fusarium* sp. and its vector tea shot hole borer (*Euwallacea fornicatus*) causing Fusarium dieback on avocado in California. *Plant Disease* **96**: 1070-1070.

Eskalen A, Stouthamer R, Lynch SC, Rugman-Jones PF, Twizeyimana M, Gonzalez A & Thibault T (2013) Host range of Fusarium dieback and its ambrosia beetle (Coleoptera: Scolytinae) vector in southern California. *Plant Disease* **97**: 938-951.

Flower CE, Dalton JE, Knight KS, Brikha M & Gonzalez-Meler MA (2015) To treat or not to treat: diminishing effectiveness of emamectin benzoate tree injections in ash trees heavily infested by emerald ash borer. *Urban Forestry & Urban Greening* **14**: 790-795.

Frago E, Dicke M & Godfray HCJ (2012) Insect symbionts as hidden players in insect–plant interactions. *Trends in Ecology & Evolution* **27**: 705-711.

Freeman S, Protasov A, Sharon M, Mohotti K, Eliyahu M, Okon-Levy N, Maymon M & Mendel Z (2012) Obligate feed requirement of *Fusarium* sp. nov., an avocado wilting agent, by the ambrosia beetle *Euwallacea* aff. *fornicata*. *Symbiosis* **58**: 245-251.

Freeman S, Sharon M, Maymon M, Mendel Z, Protasov A, Aoki T, Eskalen A & O'Donnell K (2013) *Fusarium euwallaceae* sp. nov.—a symbiotic fungus of *Euwallacea* sp., an invasive ambrosia beetle in Israel and California. *Mycologia* **105**: 1595-1606.

Freeman S, Sharon M, Dori-Bachash M, Maymon M, Belausov E, Maoz Y, Margalit O, Protasov A & Mendel Z (2016) Symbiotic association of three fungal species throughout the life cycle of the ambrosia beetle *Euwallacea* nr. *fornicatus*. *Symbiosis* **68**: 115-128.

García-Avila CDJ, Trujillo-Arriaga FJ, López-Buenfil JA, González-Gómez R, Carrillo D, Cruz LF, Ruiz-Galván I, Quezada-Salinas A & Acevedo-Reyes N (2016) First report

of *Euwallacea* nr. *fornicatus* (Coleoptera: Curculionidae) in Mexico. *Florida Entomologist* **99**: 555-557.

Gomez DF, Skelton J, Steininger MS, Stouthamer R, Rugman-Jones P, Sittichaya W, Rabaglia RJ & Hulcr J (2018) Species delineation within the *Euwallacea fornicatus* (Coleoptera: Curculionidae) complex revealed by morphometric and phylogenetic analyses. *Insect Systematics and Diversity* **2**: 2.

Grosman DM, Eskalen A & Brownie C (2019) Evaluation of Emamectin Benzoate and Propiconazole for Management of a New Invasive Shot Hole Borer (*Euwallacea* nr. *fornicatus*, Coleoptera: Curculionidae) and Symbiotic Fungi in California Sycamores. *Journal of Economic Entomology* **112:** 1267-1273.

Guadet J, Julien J, Lafay JF & Brygoo Y (1989) Phylogeny of some *Fusarium* species, as determined by large-subunit rRNA sequence comparison. *Molecular Biology and Evolution* **6**: 227-242.

Gubler DJ (1998) Resurgent vector-borne diseases as a global health problem. *Emerging Infectious Diseases* **4**: 442.

Guevara-Avendaño E, Carrillo JD, Ndinga-Muniania C, Moreno K, Méndez-Bravo A, Guerrero-Analco JA, Eskalen A & Reverchon F (2018) Antifungal activity of avocado rhizobacteria against *Fusarium euwallaceae* and *Graphium* spp., associated with *Euwallacea* spp. nr. *fornicatus*, and *Phytophthora cinnamomi*. *Antonie van Leeuwenhoek* **111**: 563-572.

Gullino ML, Fletcher J, Gamliel A & Stack JP (2008) *Crop biosecurity: assuring our global food supply*. Springer Science & Business Media.

Harris KF & Maramorosch K (2013) *Vectors of plant pathogens*. Academic Press.

Holmes BJ (2008) Communicating about emerging infectious disease: The importance of research. *Health, Risk & Society* **10**: 349-360.

Huet G (2014) Breeding for resistances to *Ralstonia solanacearum*. *Frontiers in Plant Science* **5**: 715.

Hughes M, Shin K, Eickwort J & Smith J (2012) First report of laurel wilt disease caused by *Raffaelea lauricola* on silk bay in Florida. *Plant Disease* **96**: 910-910.

Hulcr J & Stelinski LL (2017) The ambrosia symbiosis: from evolutionary ecology to practical management. *Annual Review of Entomology* **62**: 285-303.

Humble LM, Alfaro RI, Gonzalez P, Villaverde R & Allegro G (2007) The threat of the ambrosia beetle *Megaplatypus mutatus* (*Chapuis*) (=*Platypus mutatus Chapuis*) to world poplar resources. *Forestry: An International Journal of Forest Research* **80**: 471-479.

Inch S & Ploetz R (2012) Impact of laurel wilt, caused by *Raffaelea lauricola,* on xylem function in avocado, *Persea americana*. *Forest Pathology* **42**: 239-245.

Ingwell LL, Eigenbrode SD & Bosque-Pérez NA (2012) Plant viruses alter insect behavior to enhance their spread. *Scientific Reports* **2**: 578.

Jones ME & Paine TD (2015) Effect of chipping and solarisation on emergence and boring activity of a recently introduced ambrosia beetle (*Euwallacea* sp., Coleoptera: Curculionidae: Scolytinae) in southern California. *Journal of Economic Entomology* **108**: 1852-1859.

Jones ME & Paine TD (2018) Potential pesticides for control of a recently introduced ambrosia beetle (*Euwallacea* sp.) in southern California. *Journal of Pest Science* **91**: 237-246.

Jones ME, Kabashima J, Eskalen A, Dimson M, Mayorquin JS, Carrillo JD, Hanlon CC & Paine TD (2017) Evaluations of insecticides and fungicides for reducing attack rates of a new invasive ambrosia beetle (*Euwallacea* sp., Coleoptera: Curculionidae: Scolytinae) in infested landscape trees in California. *Journal of Economic Entomology* **110**: 1611-1618.

Kasson MT, O'Donnell K, Rooney AP, Sink S, Ploetz RC, Ploetz JN, Konkol JL, Carrillo D, Freeman S & Mendel Z (2013) An inordinate fondness for *Fusarium*: phylogenetic diversity of *Fusaria* cultivated by ambrosia beetles in the genus *Euwallacea* on avocado and other plant hosts. *Fungal Genetics and Biology* **56**: 147-157.

Khadempour L, LeMay V, Jack D, Bohlmann J & Breuil C (2012) The relative abundance of mountain pine beetle fungal associates through the beetle life cycle in pine trees. *Microbial Ecology* **64**: 909-917.

Kirkendall LR (2017) *New Synonymies in Neotropical Platypodinae (Coleoptera: Curculionidae)*. SPIE.

Kirkendall LR, Biedermann PH & Jordal BH (2015) Evolution and diversity of bark and ambrosia beetles. *Bark Beetles* 85-156. Elsevier.

Kress WJ, García-Robledo C, Uriarte M & Erickson DL (2015) DNA barcodes for ecology, evolution, and conservation. *Trends in Ecology & Evolution* **30**: 25-35.

Kühnholz S, Borden JH & Uzunovic A (2001) Secondary ambrosia beetles in apparently healthy trees: adaptations, potential causes and suggested research. *Integrated Pest Management Reviews* **6**: 209-219.

Laluk K & Mengiste T (2010) Necrotroph attacks on plants: wanton destruction or covert extortion? *The Arabidopsis Book/American Society of Plant Biologists* **8**: 5-19.

Leach JG (1940) Insect transmission of plant diseases. *Insect Transmission of Plant Diseases*.

Leslie JF & Summerell BA (2008) *The Fusarium Laboratory Manual*. John Wiley & Sons.

Liu X, Sievert J, Arpaia ML & Madore MA (2002) Postulated physiological roles of the seven-carbon sugars, mannoheptulose, and perseitol in avocado. *Journal of the American Society for Horticultural Science* **127**: 108-114.

Lounibos LP (2002) Invasions by insect vectors of human disease. *Annual Review of Entomology* **47**: 233-266.

Lynch SC, Twizeyimana M, Mayorquin JS, Wang DH, Na F, Kayim M, Kasson MT, Thu PQ, Bateman C & Rugman-Jones P (2016) Identification, pathogenicity and abundance of *Paracremonium pembeum* sp. nov. and *Graphium euwallaceae* sp. nov.—two newly discovered mycangial associates of the polyphagous shot hole borer (*Euwallacea* sp.) in California. *Mycologia* **108**: 313-329.

Macdonald G (1957) The epidemiology and control of malaria. *The Epidemiology and Control of Malaria*. Oxford University Press, London.

Mayer CJ, Vilcinskas A & Gross J (2008) Phytopathogen lures its insect vector by altering host plant odor. *Journal of Chemical Ecology* **34**: 1045-1049.

Mellor PS & Hamblin C (2004) African horse sickness. *Veterinary Research* **35**: 445-466.

Mendel Z, Protasov A, Sharon M, Zveibil A, Yehuda SB, O'Donnell K, Rabaglia R, Wysoki M & Freeman S (2012) An Asian ambrosia beetle *Euwallacea fornicatus* and

its novel symbiotic fungus *Fusarium* sp. pose a serious threat to the Israeli avocado industry. *Phytoparasitica* **40**: 235-238.

Moore N, Pegg K, Bentley S & Smith L (2001) Fusarium wilt of banana: global problems and perspectives. *International Workshop on the Banana Fusarium Wilt Disease.*

Muis A (2016) Biomass production and formulation of *Bacillus subtilis* for biological control. *Indonesian Journal of Agricultural Science* **7:** 51-57

Nel M & De Lange J (1985) Shoot tip grafting of avocado for virus and viroid elimination. *South African Avocado Growers Association Yearbook* **8**: 66-69.

Nuttall P, Paesen G, Lawrie C & Wang H (2000) Vector-host interactions in disease transmission. *Journal of Molecular Microbiology and Biotechnology* **2**: 381-386.

O'Donnell K (1996) Progress towards a phylogenetic classification of *Fusarium. Sydowia* **48**: 57-70.

O'Donnell K, Ward TJ, Robert VA, Crous PW, Geiser DM & Kang S (2015) DNA sequence-based identification of *Fusarium*: current status and future directions. *Phytoparasitica* **43**: 583-595.

O'Donnell K, Sink S, Libeskind-Hadas R, Hulcr J, Kasson MT, Ploetz RC, Konkol JL, Ploetz JN, Carrillo D & Campbell A (2015) Discordant phylogenies suggest repeated

host shifts in the *Fusarium–Euwallacea* ambrosia beetle mutualism. *Fungal Genetics and Biology* **82**: 277-290.

Owens D, Kendra PE, Tabanca N, Narvaez TI, Montgomery WS, Schnell EQ & Carrillo D (2018) Quantitative analysis of contents and volatile emissions from α-copaene and quercivorol lures, and longevity for attraction of *Euwallacea* nr. *fornicatus* in Florida. *Journal of Pest Science* **92**: 237-252.

Paap T, de Beer ZW, Migliorini D, Nel WJ & Wingfield MJ (2018) The polyphagous shot hole borer (PSHB) and its fungal symbiont *Fusarium euwallaceae*: a new invasion in South Africa. *Australasian Plant Pathology* **47**: 231-237.

Perrings C, Dehnen-Schmutz K, Touza J & Williamson M (2005) How to manage biological invasions under globalization. *Trends in Ecology & Evolution* **20**: 212-215.

Petersen LR, Jamieson DJ, Powers AM & Honein MA (2016) Zika virus. *New England Journal of Medicine* **374**: 1552-1563.

Ploetz R, Pérez-Martínez J, Smith J, Hughes M, Dreaden T, Inch S & Fu Y (2012) Responses of avocado to laurel wilt, caused by *Raffaelea lauricola*. *Plant Pathology* **61**: 801-808.

Ploetz RC, Hulcr J, Wingfield MJ & De Beer ZW (2013) Destructive tree diseases associated with ambrosia and bark beetles: black swan events in tree pathology? *Plant Disease* **97**: 856-872.

Popa V, Déziel E, Lavallée R, Bauce E & Guertin C (2012) The complex symbiotic relationships of bark beetles with microorganisms: a potential practical approach for biological control in forestry. *Pest Management Science* **68**: 963-975.

Purcell AH (1982) Insect vector relationships with procaryotic plant pathogens. *Annual Review of Phytopathology* **20**: 397-417.

Purcell J, Brelsford A & Avilés L (2012) Co-evolution between sociality and dispersal: the role of synergistic cooperative benefits. *Journal of Theoretical Biology* **312**: 44-54.

Ranger CM, Schultz PB, Frank SD, Chong JH & Reding ME (2015) Non-native ambrosia beetles as opportunistic exploiters of living but weakened trees. *PLoS One* **10**: e0131496.

Rogers N (1989) Germs with legs: flies, disease, and the new public health. *Bulletin of the History of Medicine* **63**: 599-617.

Rugman-Jones P & Stouthamer R (2016) Polyphagous-and Kuroshio shot hole borers: invasive *Euwallacea* spp. threatening Californian agriculture and natural areas. *Laurel Wilt Summit, University of Florida IFAS Tropical Research and Education Center* 3-4.

Rust MK & Su N-Y (2012) Managing social insects of urban importance. *Annual Review of Entomology* **57**: 355-375.

Scholthof K-BG (2007) The disease triangle: pathogens, the environment and society. *Nature Reviews Microbiology* **5**: 152.

Scora RW & Bergh B (1989) The origins and taxonomy of avocado (*Persea americana* Mill.) Lauraceae 387-394. Springer.

Scudder GG (2009) The importance of insects. *Insect biodiversity: science and society* **1**: 7-32. Elsevier.

Short DP, O'Donnell K, Stajich JE, Hulcr J, Kijimoto T, Berger MC, Macias AM, Spahr EJ, Bateman CC & Eskalen A (2017) PCR multiplexes discriminate *Fusarium* symbionts of invasive *Euwallacea* ambrosia beetles that inflict damage on numerous tree species throughout the United States. *Plant Disease* **101**: 233-240.

Sisterson MS (2008) Effects of insect-vector preference for healthy or infected plants on pathogen spread: insights from a model. *Journal of Economic Entomology* **101**: 1-8.

Six DL (2003) A comparison of mycangial and phoretic fungi of individual mountain pine beetles. *Canadian Journal of Forest Research* **33**: 1331-1334.

Six DL (2012) Ecological and evolutionary determinants of bark beetle—fungus symbioses. *Insects* **3**: 339-366.

Smith S, Gomez D, Beaver R, Hulcr J & Cognato A (2019) Reassessment of the species in the *Euwallacea fornicatus* (Coleoptera: Curculionidae: Scolytinae) complex after the rediscovery of the “lost” type specimen. *Insects* **10**: 261.

Stahl EA & Bishop JG (2000) Plant–pathogen arms races at the molecular level. *Current opinion in Plant Biology* **3**: 299-304.

Stouthamer R, Rugman-Jones P, Thu PQ, Eskalen A, Thibault T, Hulcr J, Wang L-J, Jordal BH, Chen C-Y & Cooperband M (2017) Tracing the origin of a cryptic invader: phylogeography of the *Euwallacea fornicatus* (Coleoptera: Curculionidae: Scolytinae) species complex. *Agricultural and Forest Entomology* **19**: 366-375.

Tautz D, Arctander P, Minelli A, Thomas RH & Vogler AP (2003) A plea for DNA taxonomy. *Trends in Ecology & Evolution* **18**: 70-74.

Thorp TG & Sedgley M (1993) Architectural analysis of tree form in a range of avocado cultivars. *Scientia Horticulturae* **53**: 85-98.

Toki W, Tanahashi M, Togashi K & Fukatsu T (2012) Fungal farming in a non-social beetle. *PLoS One* **7**: e41893.

Umeda C, Eskalen A & Paine TD (2016) Polyphagous shot hole borer and Fusarium dieback in California. *Insects and Diseases of Mediterranean Forest Systems* **6**: 757-767. Springer.

van den Berg N, du Toit M, Morgan S, Fourie G & de Beer ZW (2019) First report of *Fusarium euwallaceae* causing necrotic lesions on *Persea americana* in South Africa. *Plant Disease* **10**: 10-18-1818-PDN.

van Diepeningen AD & de Hoog GS (2016) Challenges in *Fusarium*, a trans-kingdom pathogen. *Mycopathologia* **181**: 161-163.

Vega FE & Hofstetter RW (2014) *Bark beetles: biology and ecology of native and invasive species*. Academic Press.

Wood SL (1982) The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. *The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph*. Springer.

Chapter 2: Beetles and accompanying fungi, associated with damage to avocado trees and the first report of *Fusarium euwallaceae* on avocado, in South Africa

TABLE OF CONTENTS

Results

ABSTRACT

The polyphagous shot hole borer (*Euwallacea fornicatus)*, ambrosia beetle is a generalist, insect species from South East Asia, considered to be an invasive pest, due to its ability to damage healthy trees. The beetle causes damage by acting as a vector to inoculate pathogenic, ambrosial fungi, into reproductive and non-reproductive plant hosts, causing Fusarium dieback. This invasive beetle and its fungal symbiont, specifically *Fusarium euwallaceae,* form a complex that is internationally notorious for the damage it has caused to a variety of plants, specifically to *Persea americana* (avocado), in Israel and California, in the United States of America. In a recent study in South Africa, this ambrosia beetle-fungal complex was detected, damaging *Platanus x acerifolia* (London Plane) trees in the KwaZulu-Natal National Botanical Gardens, Pietermaritzburg. This observation prompted rising concern by the local avocado growing communities. The aims of this study were to survey and identify the beetles associated with/found on diseased avocado plants and to isolate and identify symbionts and/or fungal pathogens connected to beetle-associated damage on avocado plants, in South Africa. In this study, several objectives were completed and included the surveying of commercial avocado orchards, as well as residential avocado trees for the presence of wood-boring beetles and fungi associated with their damage. This was done to gauge the spread and prevalence of Fusarium Dieback, on avocado, in South Africa, as well as to detect the presence of one or more of the PSHB fungal symbionts. As part of this survey, a variety of isolation techniques were used and led to the detection of the PSHB and an accompanying fungal organism, causing damage to an avocado tree, in Sandton, Johannesburg. The objective was then to molecularly and phylogenetically identify the fungal organism associated with the disease. Once identified as *F. euwallaceae*,

Koch's postulates were carried out to determine the causal agent for the observed disease and to confirm the pathogenicity of *F. euwallaceae* to avocado. The impact hereof drew further attention to the local introduction of this pest complex and the degree of threat posed to local agriculture, specifically to that of the avocado industry.

INTRODUCTION

The introduction and establishment of an agricultural pest, in an area that it has not previously occupied, can have devastating effects on local plant, human and animal communities (Kolar & Lodge, 2001). The repercussion of such an introduction can impact the economy as well as the viability, sustainability and feasibility to cultivate certain valued or desired plant species, within an area (White *et al.*, 2006). A lack of stringent national border security and biosecurity can sometimes allow for the accidental introduction of micro-organisms or insects from foreign countries. Unlike the slowly-rising threat posed by pathogenic micro-organisms, the threat posed by newly introduced insect pests, particularly insects that can disperse autonomously, can be largely attributed to their ability to spread geographically, despite the application of control measures and practices (Perrings *et al.*, 2005).

Pathogen spread occurs via many routes, but their rate and extent of dispersal can be accelerated by the presence of an enabling biological entity, such as an insect vector (Perrings *et al.*, 2005). One group of insect vectors, that has historically posed a threat to forestry biosecurity and agriculture, are the ambrosia beetles (Eskalen *et al.*, 2013). These insects inoculate their pathogenic, symbiotic fungi, directly into plant tissue, thereby causing disease. The polyphagous shot hole borer (*Euwallacea fornicatus* Eichhoff.) is one such ambrosia beetle, that has a symbiotic relationship with several fungal organisms (Freeman *et al.*, 2013). This insect causes damage by inoculating

these pathogenic fungi into reproductive and non-reproductive plant hosts, causing Fusarium Dieback (FD) (Eskalen *et al.*, 2012). This invasive beetle and its fungal symbiont, specifically *Fusarium euwallaceae* S. Freeman, Z. Mendel, T. Aoki & O'Donnell*.,* form a complex that is internationally notorious for the damage it has caused to trees, specifically on avocado (*Persea americana* Mill.) (Eskalen *et al.*, 2012, Mendel *et al.*, 2012, Umeda *et al.*, 2016).

As a means of effective surveillance and early detection, the status and prevalence of such invasive insects, are often monitored using sentinel projects or programmes (Vettraino *et al.*, 2015). This is whereby selected plant species are grown and monitored, in specific regions and used as tools to indicate the incidence and the degree of damage that could be inflicted on highly-valued plant species, given the presence of an internationally invasive pest species. During a local sentinel project, initially established to monitor tree health, in the KwaZulu-Natal National Botanical Gardens, of South Africa, an ambrosia beetle, as well as a fungal associate, was detected, causing disease on *Platanus x acerifolia* (London Plane) trees (Paap *et al.*, 2018). Subsequent genetic analyses revealed the identity of the internationally invasive ambrosia beetle as *E. fornicatus* (H33) or polyphagous shot hole borer (PSHB). Alongside its presence, the pathogenic fungal symbiont – *F. euwallaceae* was also isolated from the diseased material. This occurrence marked the first report of this beetle-fungus complex causing FD in South Africa and by the end of the first year, since the first report of this beetle-fungus complex, the spread of the pest had progressed significantly.

Given the international notoriety for damage that this pest has caused and a critical lack of awareness of the local public, the Forestry and Agricultural Biotechnology

Institute (FABI) at the University of Pretoria, launched several studies to conduct basic research and to monitor and survey for the presence of this disease. Field excursions were conducted to collect diseased material and associated beetles in several different avocado growing areas, such as Tzaneen, in Limpopo, White River, Kiepersol, Hazyview, in Mpumalanga, Howick, in KwaZulu-Natal and Sandton, in Gauteng. The sampling of the diseased tissue and live beetles included primary isolations that were performed onto amended and unamended nutrient media. The intention hereof being to detect the vector organism (*E. fornicatus)* and/or one or more of its fungal associates. In addition, by creating awareness, samples were also received from concerned members of the public, particularly avocado farmers. Several beetles and fungal cultures were also obtained from the Avocado Research Programme (ARP), as a result of collecting and screening beetles, associated with disease avocadoes, in the past (from March 2016 until September 2019).

In order to determine the identity of the unknown organisms, obtained from the isolation of diseased avocado tissue, a combination of detection techniques and tools were applied. The objectives of which included the use of different isolation techniques to detect for ambrosia-beetle-related fungi as well as microscopy and morphologic studies to exclude environmentally-abundant, non-pathogenic fungal isolates from downstream study. Molecular identification was carried out based on amplifying several universally conserved fungal genes regions (*TEF1α, β-t* and *RBP2*) and phylogenetic inference of the partial gene sequences was used to determine species identities and to distinguish closely related species from one another. The objectives were carried out successfully, allowing for the detection of several ambrosia beetles and fungi, as well as the presence of the PSHB and its pathogenic associate (*F. euwallaceae*), on avocado.

MATERIALS AND METHODS

Primary isolations and beetle collection from diseased avocado lesions

To isolate and study *F. euwallaceae* and/or other fungal micro-organisms, associated with disease symptoms of FD, half-strength potato dextrose agar (hereafter ½ PDA) and Fusarium spp. selective medium (hereafter FSM) were prepared. ½ PDA nutrient medium was prepared by adding 19.5 g PDA, 7.5 g BD™ Difco™ agar and distilled water to a volume of 1 L. The selective medium (FSM) was prepared by adding 15 g of peptone, 1 g of potassium phosphate (KH_2PO_4), 0.5 g magnesium sulphate ($MgSO_4$.$7H_2O$), 20 g BD™ Difco™ agar, 1 g Pentachloronitrobenzene (PCNB) in 5 ml acetone, 1 mg Streptomycin dissolved in 3 ml water and distilled water to a volume of 1 L. The heat-sensitive chemicals, used to amend the FSM, were added once the medium had cooled down to 55°C.

Primary isolations were conducted in both field- and laboratory conditions, from live beetles, beetle holes and the associated disease lesions, on avocado tissue. The major avocado growing provinces in the North Eastern region of South Africa (Limpopo and Mpumalanga) and KwaZulu-Natal were repeatedly sampled, from early 2016 to the middle of 2019. During sampling, any observed disease symptoms consistent with insect/fungal damage were used as the basis for further study and isolations were performed from Hass, Fuerte, Edranol, Pinkerton and Ryan avocado cultivars. A rubber hammer and metal-tipped chisel were used to strip away the bark on symptomatic avocado plant tissue, in order to reveal the underlying lesions. A scalpel blade was disinfected in ethanol and flamed. Small blocks (5 mm x 5 mm) were cut from the border of the lesions. These blocks were plated, in replicates, onto the surface of both ½ PDA and FSM. Similarly, any live or dead beetles found in proximity to

beetle-damaged avocado trees were placed onto the surface of both media types using entomological forceps, taking care not to damage the insects. The insects were allowed to crawl around freely on the medium for approximately 5 min, or if they remained sedentary, they were gently streaked across the surface of the nutrient medium, using forceps, in an attempt to isolate any fungal symbionts. The Petri-plates were incubated in the dark, at 25°C, for 5 – 7 days or until fungal colonies started to appear. The colonies were morphologically analysed with a light microscope (Zeiss SteREO Discovery V8), after incubation. The resulting fungal cultures of interest were then transferred and sub-cultured onto ½ PDA for purification. After using the live beetle samples for primary isolations, the live and dead beetles were placed into 100% absolute ethanol and stored at -20°C. Dead beetle samples were also collected from ethanol traps and other potential PSHB hosts plants around the avocado orchards and stored in the same way.

Identification of purified fungal cultures from primary isolations

DNA extraction from fungal cultures

To identify the unknown fungal organisms from the primary isolations, DNA extraction using the PrepMan® extraction protocol was performed on each of the purified fungal cultures. 50 mg of mycelia, was transferred to individual 1.5 ml Eppendorf tubes, using a heat-sterilised needle tip, along with 50 µl PrepMan® Ultra Reagent (Applied Biosystems) per tube. The samples were incubated on a heating-block for 5 min at 96°C. Thereafter, the mixtures were ground up/homogenised in the Eppendorf tubes, using a flamed pipette tip as a pestle, and were mixed using a vortex and incubated at 96°C for 10 min. The samples were centrifuged, at 10 000 rpm for 5 min and the resulting supernatants were carefully transferred to new tubes. To each supernatant,

80 µl of 10 mM Tris-HCl pH 8 was added and then stored at -20°C, thereby yielding the total genomic DNA. Once DNA was extracted, the cultures were re-plated and stored at 4°C.

PCR amplification of DNA barcode regions for identification of fungal isolates

The PCR protocol used for all the conventional amplifications involved adding together 1 U FastStart Taq DNA Polymerase, 2.5 µl 10X PCR reaction buffer, 200 µM of each dNTP, 10 µM of forward and 10 µM of reverse primer, 2 µl template DNA (100 ng/µl) and sterile water to a total volume of 25 µl. Each PCR performed was accompanied with a negative control, containing PCR grade water instead of template DNA, to ensure the absence of contamination from the reactions. The mixture of components was placed in an Eppendorf Thermocycler, using the relevant thermal programmes.

The fungal barcode gene selected as the target, for the PCR amplification and identification of all the fungal cultures, was the translation elongation factor 1α (*TEF1*α) gene region, using *Fusarium* spp. specific primer set (EF1/EF2). The thermal conditions were 5 min at 95°C for initial denaturation, 40 cycles of 1 min at 94°C, 1 min at 67°C, and 1 min at 72°C for annealing, and then a final 10 min at 72°C for the final extension stage. The holding temperature was set to 4°C.

To confirm the identity of isolates suspected to be *F. euwallaceae,* subsequent PCR amplification of the β-tubulin (*β-t*) and RNA polymerase II second largest subunit (*RBP2*) gene regions were also performed. The thermal conditions for the PCR, for the *β-tubulin* gene region were 5 min at 95°C for initial denaturation, 30 cycles of 30 sec at 95°C, 1 min at 54°C, and 1 min at 72°C for annealing, and then a final 10 min at 72°C for the final extension stage. The thermal conditions for the PCR, for the *RBP2*

gene region were 1 min at 95°C for initial denaturation, 25 cycles of 45 sec at 95°C, 40 sec at 52°C, and 2 min at 72°C for annealing. This was followed by a further 15 cycles of 45 sec at 95°C and 40 sec at 52°C and then 2 min at 72°C for the final extension stage. Primer sequences, used to amplify each gene region, are indicated in **Table 1**, in the supplementary information.

Agarose gel electrophoresis of products from *TEF1α* gene PCR

The products from the PCR amplifications were analysed by 2% agarose gel (Seakem®) electrophoresis to ensure successful amplification of the DNA. 2 µl loading dye was added to 5 µl PCR product and then loaded into individual wells, alongside a Quick-Load 100 bp DNA Ladder (Thermofisher Scientific). An electrophoretic tank (BioRad Basic) was set to 80 V and current was allowed to flow for 5 min and then for a further 25 min at 110 V. The resultant gel was visualised under a UV transilluminator.

Sequencing PCR of fungal PCR products

In order to enzymatically purify the PCR products, the EXO-SAP reagent was prepared. 5 µl of Exonuclease I (20 u/µl) and 100 µl of Shrimp alkaline phosphatase (1 u/µl) were added to distilled water to a volume of 1 L. 2 µl of EXO-SAP were added to every 5 µl of PCR product. Once the EXO-SAP reagent was added, the samples were mixed and were incubated in a thermal cycler for 15 min at 37°C and then for 15 min at 80°C. The purified samples were stored at 4°C.

For each purified sample, two separate amplifications were carried out for bi-directional sequencing of the PCR products. One reaction containing the forward primer and the other containing the reverse primer. Each reaction contained 2 µl purified PCR product, 10 mM primer (**EF1** or **EF2**), 0.5 µl BigDye (ThermoFisher

Scientific), 2.1 µl 5X Sequencing Buffer (ThermoFisher Scientific) and 6.4 µl PCR grade water. The thermal program followed initial denaturation at 96°C for 2 min and 30 cycles of 30 sec denaturation at 94°C, 15 sec annealing at 55°C and 4 minute extension at 60°C, followed by a holding temperature of 4°C.

Precipitation of the products from the sequencing PCR involved adding 8 µl of sterile water, 2 µl 3M NaAc pH 5.2 and 50 µl 100% absolute ethanol to 12 µl of each sequencing-PCR product, in a 1 ml Eppendorf tube. The solutions were mixed using a vortex and were incubated at 4°C for 10 min. Thereafter, the samples were centrifuged at 12 000 rpm for 30 min and the resulting supernatants were discarded. The remaining pellets were washed, twice, with 70% ethanol and once the ethanol was removed, the tubes were left to air dry, in a sterile environment, for approximately 20 min. The products were submitted for sequencing at the DNA Sanger sequencing facility in the Faculty of Natural and Agricultural Sciences, University of Pretoria (UID:78566).

Raw sequence data editing, contig assembly, multiple sequence alignment and *Fusarium euwallaceae* haplotype determination

The forward and reverse sequences of the fungal isolates were edited using the BioEdit sequence alignment editor. The sequence edges were trimmed and ambiguous nucleotides were edited. The forward and reverse sequences were assembled into contigs and were then submitted to BLASTx analyses. All sequences that did not show association with the PSHB or its fungal symbionts, from the BLAST analysis, were recorded and excluded from further study. To study the remaining sequences, a database containing the publicly available sequences, of the organism of interest (*F. euwallaceae*), was created, using MEGA 7 software. The unknown

sequences of interest were imported into the database and a multiple sequence alignment (MSA) was performed, using MAFFT 7 online. The MSA consisted of *TEF1*α sequences from *F. euwallaceae* isolates, that were obtained from various areas and hosts around the world and included the sequences for the London Plane isolates from KwaZulu-Natal National Botanical Gardens, in South Africa, as well as sequences of *F. euwallaceae* isolates obtained from avocado plants, affected with FD, in Israel and California. Sequences for MSA obtained from Eskalen *et al.* (2012), O'Donnell *et al.* (2015), Na *et al.* (2018) and Paap *et al.* (2018).

In addition to confirming the identity of the unknown sequences, sequences that represented *F. euwallaceae* isolates were further scrutinised for the detection of potentially novel haplotypes. The haplotypes were determined by considering intron splice sites on an annotated map, constructed by Yin *et al.* (2015), for the complete translation elongation factor 1 alpha gene, based on the genome (GL629769) of *Grosmannia clavigera* (isolate kw1407), sequenced by DiGuistini *et al.* (2011). The introns were trimmed at the AG/GT intron splice sites from all the sequences and single-nucleotide comparisons were done to the other sequences in the MSA, to determine if any novel haplotypes were present.

Phylogenetic tree construction and identity inference of *Fusarium* spp.

Using the database, containing the sequences from the MSA, of partial *TEF1*α gene sequences, a phylogenetic framework was constructed by generating phylogenetic trees/structures, containing the sequences for the unknown organisms, relative to the known sequences. Maximum likelihood phylogenetic trees were constructed using MEGA 7 software tools with 1000 bootstraps. The structures hereof allowed for the exclusion of certain unknown organisms or confirmation of identities for others.

Koch's postulates to confirm *Fusarium euwallaceae* as the causal agent for the disease on avocado

Once the morphologic, molecular and phylogenetic analyses confirmed the identity of *F. euwallaceae,* from diseased avocado tissue, the relevant study was carried out to validate that the isolate of *F. euwallaceae* was indeed the causal agent for the observed FD disease symptoms. This process involved the successful isolation from diseased avocado plant tissue, Sandton, Johannesburg, in Gauteng and subsequent transferring onto selective medium (FSM) to obtain a pure culture of the suspected causal agent. A PSHB specimen could not be retrieved from the sampled avocado tissue, but a confirmed PSHB specimen was retrieved from a diseased Chinese Maple tree (*Acer buergerianum*) approximately 2 km away also harbouring *F. euwallaceae*, that was genetically identical to the isolate from the avocado tissue. The beetle was identified by FABI, by means of DNA extraction, PCR and sequencing of the mitochondrial cytochrome c oxidase 1 (*COX1*) gene region. The resulting sequence was submitted to the online GenBank sequence database (*COX1* - MH823819).

Once the identity of the successfully isolated culture was phylogenetically confirmed as *F. euwallaceae,* from the avocado tissue, it was used in a pathogenicity trial. Using a cork borer, shallow excisions were made from various areas of the xylem, of nine avocado plantlets about 12 months old and 75 cm tall (Hass cultivar grafted onto R 0.09 rootstock). Seven biological replicates and two control plants were used during the pathogenicity trial, with three technical replicates, per plant.

The freshly wounded tissue, of seven plantlets, was inoculated with *F. euwallaceae,* cultured on ½ PDA plugs, 3 mm in diameter. For two plants, clean ½ PDA plugs were used, instead of *F. euwallaceae* colonised plugs, as the controls. The wounds were

wrapped with parafilm and were left to incubate in a greenhouse, for 6 weeks at 25°C. The plants were watered twice per week and fertilised once a week with Wonder Nitrosol® (Efekto).

After 6 weeks of incubation, the plants were photographed, the parafilm was removed, lesions were observed and their lengths were measured. In addition to measuring the size of the lesions, primary isolations were made onto FSM, from the edges of the lesions (infected and control plants), to recover *F. euwallaceae* from the infected plants, as well as to ensure the absence of *F. euwallaceae* from the control plants. Similar results were obtained in a repeat experiment.

PrepMan® DNA extraction was then carried out on the re-isolated fungal culture and subsequent PCR amplification of the translation elongation factor 1α (*TEF1*α), β-tubulin (*β-t*), and RNA polymerase II second largest subunit (*RBP2*) gene regions were done. The thermal conditions for the PCR and primers, described on **Page 67**, were used to amplify the *TEF1*α, *β-tubulin* and *RBP2* gene regions. The resulting sequences were submitted to the online GenBank sequence database (*TEF1*α - MH823818), (*β-tubulin* - MH823816), (*RBP2* - MH823817).

The initial isolation, successful infection and ultimate re-isolation from the artificially inoculated plants fulfilled Koch's postulates and concluded that *F. euwallaceae* was the cause of the disease. Analysis of the data determined the normality distribution and a one-way analysis of variance (ANOVA), using GraphPad Prism 8, was done on the mean lesion lengths, of the lesions/wounds from the pathogenicity trial. Further analysis was done to determine the standard error and whether the mean lesion lengths of the infected plants differed significantly from that of the mean lesion lengths of the control plants.

Identification of beetles from avocado orchards

Morphologic analysis of beetles for identification

Before attempting to conduct any downstream molecular studies on the beetle samples, extensive record-keeping was done based on morphological characteristics. High-quality images were obtained, through microscopic photography for a confirmed specimen of PSHB (Zeiss SteREO Discovery Microscope using a specialised Zeiss ICC5 camera). Various angles were photographed, to be used as a comparative tool, to differentiate other wood-boring beetles from the PSHB.

Previously sampled, unknown, preserved beetles were removed from the ethanol that they were stored in and placed onto Whatman® filter paper. The beetles were then thoroughly, yet carefully, submerged and washed, twice, in distilled water. Using light microscopy and considering key morphological characteristics of the PSHB and closely related species, morphological observations allowed for the exclusion of beetles that did not resemble the PSHB. The major beetle groups that were deemed not to be the PSHB were not included in downstream molecular analyses, but they were grouped, photographed and their suspected genera were recorded. It is generally desirable to accompany the morphological identification of a species with a molecular supposition, however in many of these cases, additional molecular analyses were circumvented for the sake of specimen preservation.

DNA extractions from notable beetle specimens

Due to the hardy nature of the exoskeleton, in combination with the size of these small beetles (most beetles surveyed were < 5 mm), difficulties regarding the extraction of high-quality DNA were encountered. For this reason, different protocols, using various

reagents were carried out, towards the goal of achieving a reliable and effective extraction protocol. These DNA extraction protocols included the use of the PrepMan® extraction kit, the prepGEM® extraction kit and the universal, rapid salt-extraction reaction. Spectrophotometry, aimed at determining DNA quality or concentration, cannot be accurately applied to these reactions and agarose gel electrophoresis on the extracted DNA is not indicative of DNA quality.

PrepMan® extraction protocol

Single beetle specimens were transferred to individual 1.5 ml Eppendorf tubes and the previously described protocol was followed to extract genomic DNA.

prepGEM® extraction protocol

The second DNA extraction protocol, that was tested, used the prepGEM® reagent (ZyGEM^{TM}). Together with a single beetle specimen, 35 µl of PCR grade water, 4 µl of 10x prepGEM® Buffer, 1 µl prepGEM® reagent were added to a 1.5 ml Eppendorf tube. The beetles were homogenised as previously described. The samples were incubated in a thermal cycler for 15 min at 75°C and then for 5 min at 95°C. To clarify the DNA-containing supernatant, centrifugation was performed at 13 000 rpm for 2 min. The supernatant, containing the DNA, was transferred to a new tube and then stored at -20°C.

Universal, rapid salt-extraction protocol

The final extraction protocol carried out was modified and sourced from Aljanabi & Martinez (1997). Singe beetle samples were homogenised within 400 µl of freshly-prepared, sterile salt homogenising buffer (0.4 M NaCl 10 mM, Tris-HCl pH 8.0 and 2

mM EDTA pH 8.0). To the homogenised mixture, 40 µl of 20% sodium dodecyl sulfate (SDS) and 8 µl of 20 mg/ml proteinase K were added and were mixed well with a vortex. The sample was placed in a heating-block and incubated at 60°C, overnight. The following day, 300 µl of NaCl (6 M NaCl saturated in H_2O) was added to the mixture. The sample was mixed for 30 sec and centrifuged at 10 000 rpm for 30 min. The supernatant was transferred to a new tube. An equal volume of chilled isopropyl alcohol was then added to the mixture, prior to incubating the sample at -20°C, for 1 hour. The samples were then centrifuged at 10 000 rpm for 20 min, at 4°C. The supernatant was discarded and the resultant pellet was washed, twice, with 70% ethanol. The remaining ethanol was poured out and the pellet was left to dry, in a sterile environment, for about 15 min or until completely dry. Once dried, the pellet was resuspended in 50 µl TE buffer (1 M Tris, adjusting to pH 8 using HCl and 1 mM Ethylenediaminetetraacetic acid (EDTA)). The TE buffer, containing the DNA, was incubated at 50°C for 1 hour and thereafter stored at -20°C.

PCR amplification of a DNA barcode region for beetle specimens

The only way to detect the presence of viable DNA, relied on successful PCR amplification, on the extracted DNA. The barcode gene selected as the target, for the identification of beetles and other insects (Vrijenhoek, 1994), was the mitochondrial cytochrome c oxidase 1 (*COX1)* gene region and the LCO1490 and HCO2198 primer set was used for amplification. The thermal conditions for the PCR were 5 min at 95°C for initial denaturation, 30 cycles of 30 sec at 94°C, 30 sec at 51°C, and 30 sec at 72°C for annealing, and then a final 5 min at 72°C for the final extension stage. The holding temperature was set to 4°C. The primer sequences, used to amplify the *COX1* gene region, are indicated in **Table 1**, in the supplementary information.

RESULTS

Primary isolations from diseased avocado lesions and live beetles

The visible response to FD varies among host species. On avocado, disease symptoms that were observed in the field included branch wilting and dieback as well as very small, pinhead shaped entry/exit holes on the stems and branches. These holes were also frequently accompanied by the presence of fine, powdery refuse (frass) as a result of mechanical damage caused by the action of wood-boring beetles. Along with the perforation of the tissue, the signature symptom of FD, on avocado was also observed, namely the presence of sugary exudates that cover the entry holes, as a disease response by the infested trees. Upon removing the symptomatic bark, internal reddish-brown discolouration, alongside water-soaked lesions, were revealed. Cross-sections performed from the infested trees revealed further internal tissue damage due to the presence of natal brood galleries, as well as extensive staining, caused by the action of the various fungal organisms. No larvae or pupae were recovered from these cross-sections. The diseased plant tissues used for primary isolations are indicated in **Figure 1.**

Sub-culturing fungi from the primary isolations from several diseased tissues, onto both media types, yielded 255 pure fungal cultures. Clear differences, using the various isolation media types, were observed. The FSM allowed the growth of several fungi, morphologically suspected to be *Fusarium* spp. A large number of these fungi, obtained from the FSM, were suspected to be of the *Fusarium* genus, due to culture morphologies and microscopic observations. Some of the morphological characteristics observed as criteria for the preliminary identification, for members of

the *Fusarium* genus included a pigmentation range between yellow, white, pink and violet, as well as slender, septate macroconidia with slight dorsoventral curvatures. These are often falcate to long clavate, septate sporodchial conidia, swollen in their upper half (Freeman *et al.*, 2013). The FSM also proved to significantly reduce the amount of bacteria and the overall number of environmentally present micro-organisms, when compared to the unamended medium. The difference in results yielded by each medium are shown in **Figure 2.** In this figure, it is clear how the FSM was able to inhibit the growth of significantly more environmentally present micro-organisms, while allowing the growth for organisms resembling the expected pigmentation range, for the *Fusarium* genus.

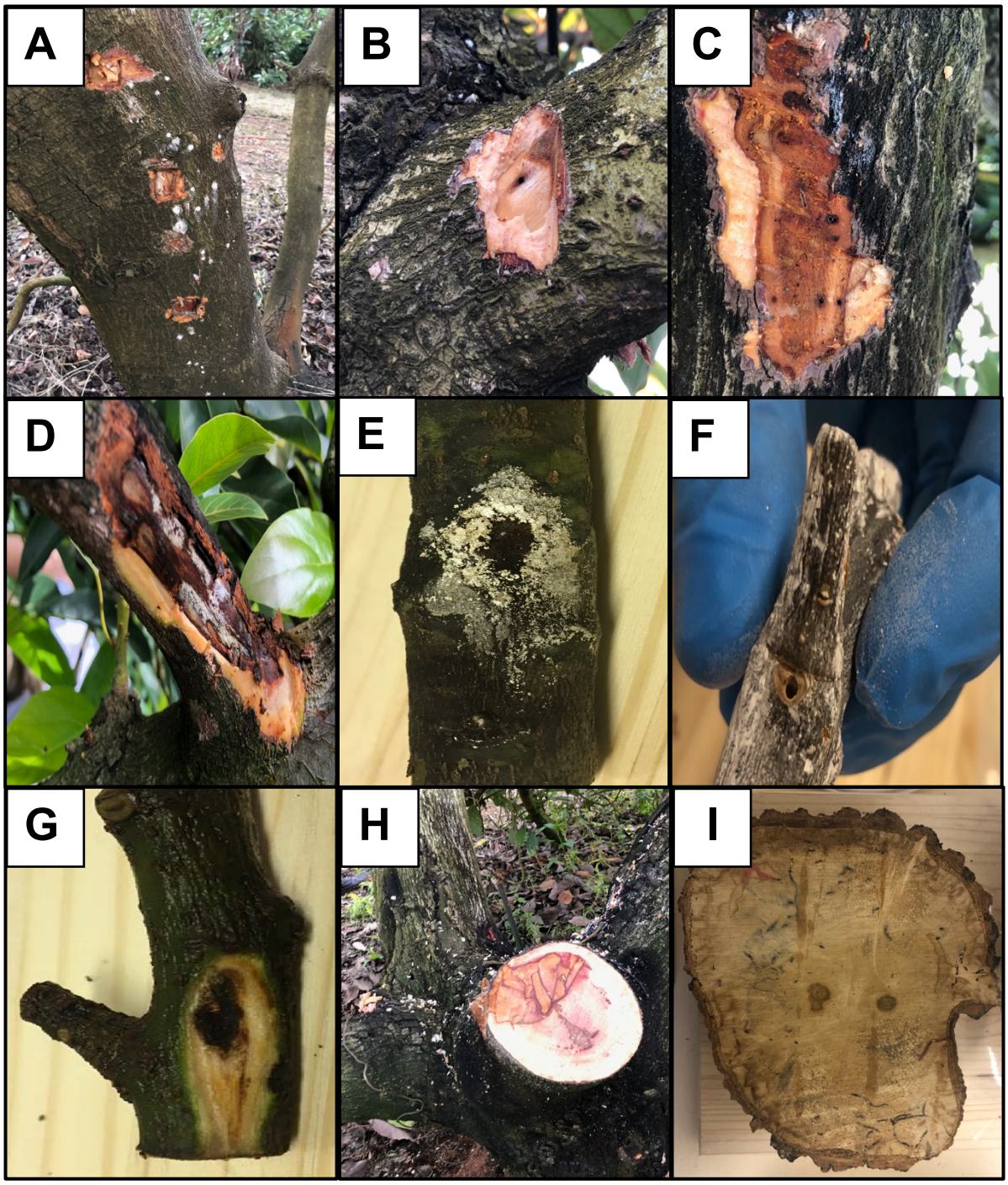


Figure 1: Symptomatic avocado plant material and diseased tissue used in this study. **A – C**: Lesions from beetle damage on avocado tree stems and branches. **A** indicates how bark was removed using a hammer and chisel. **D – G**: Branches with beetle holes and/or water-soaked lesions. **E** - White sugary exudates ("sugar volcanoes") at the point of entry. **H – I**: Cross-sections revealing extensive pink/dark-brown staining and presence of beetle galleries. The above forms of material were all used to conduct primary isolations.

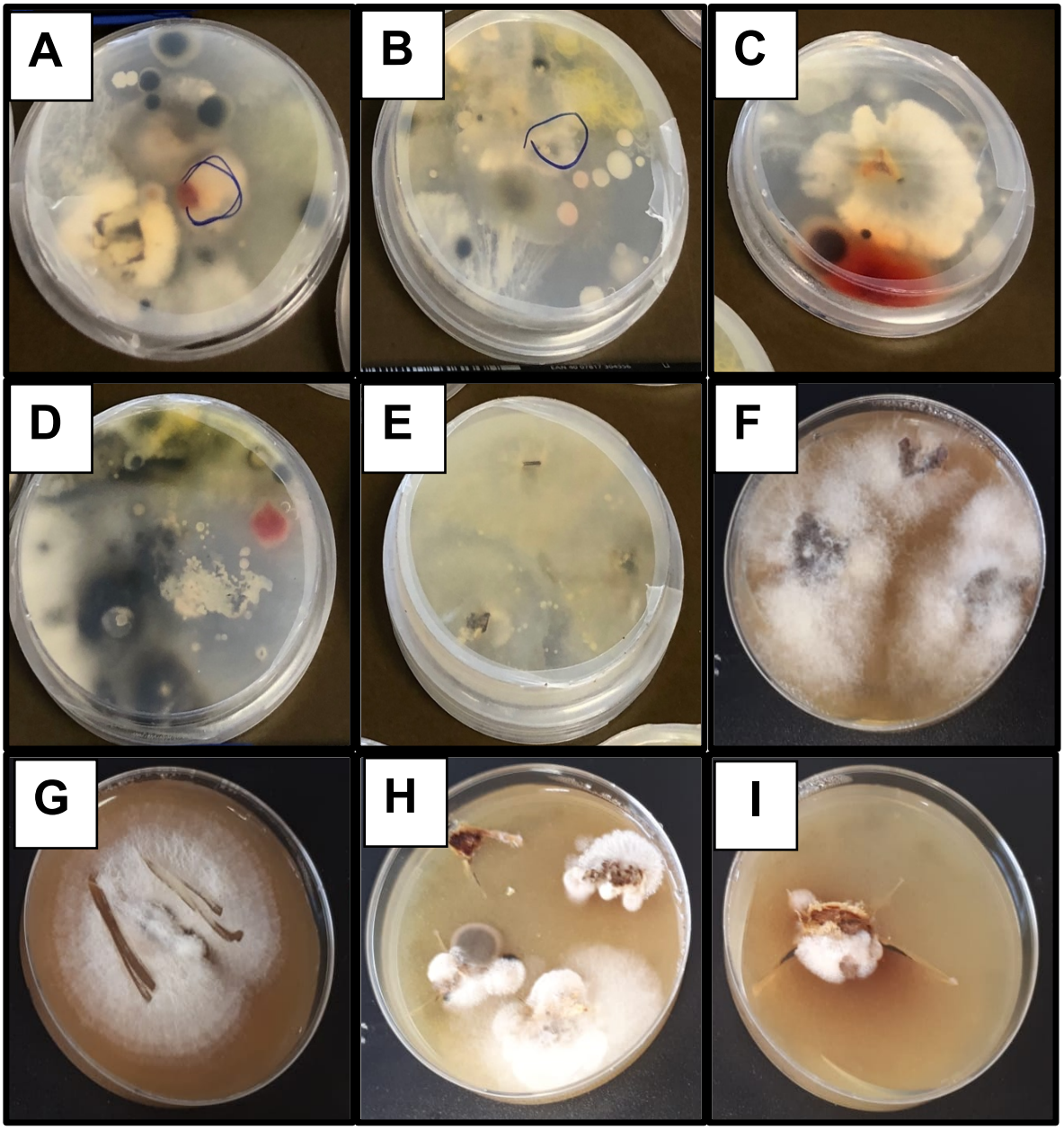


Figure 2: Results obtained from the primary isolations, from lesions on disease avocado lesions. **A – E:** Primary isolations performed onto the unamended ½ PDA, were used primarily to scout for other fungal symbionts associated with the PSHB. **F – I**: Primary isolations performed onto the chemically amended Fusarium selective medium (FSM). Primary isolations performed onto the selective medium proved more effective at limiting the growth of undesirable environmental micro-organisms, while allowing the growth of organisms resembling *Fusarium* spp., thereby increasing the chances of successfully isolating *Fusarium euwallaceae.*

Identification of purified fungal cultures from primary isolations

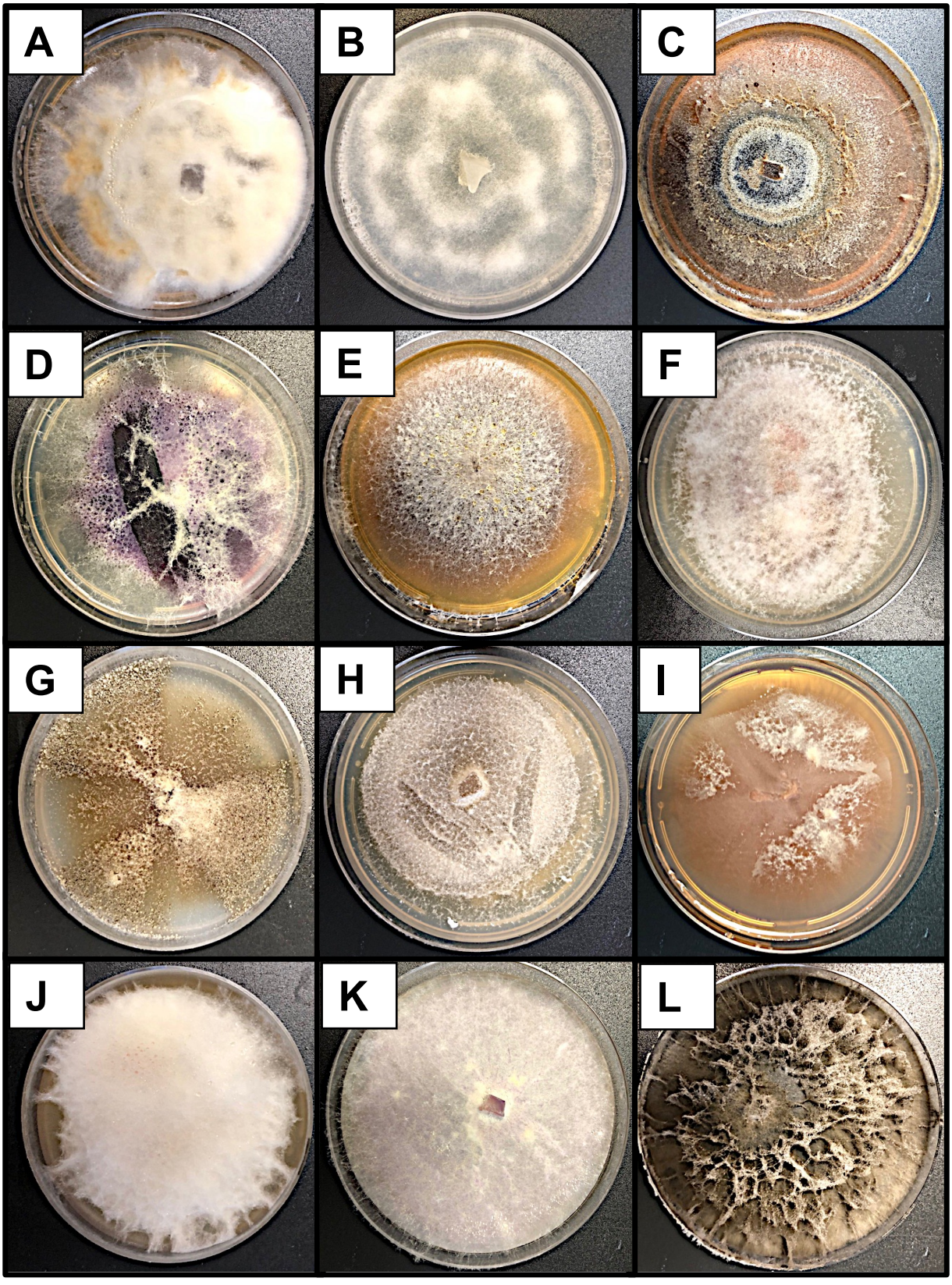
Several major morphological groups were detected before sequencing. The culture morphologies were used alongside the molecular tools in order to identify the major genera that were present. For all the unknown organisms, illustrated in **Figure 3**, identities were determined until at least genus level.

Due to the genus-specific nature of the primers that were used, samples indicated by a lack of a band, in the agarose gel, indicated that the representative isolate was not within the *Fusarium* genus. However given the use of a barcode gene, successful amplification of DNA, from non-*Fusarium* organisms did occur, but with less bright bands, in many instances. Difficulties related to contamination, indicated by unexpected bands in the agarose gel electrophoresis, in the negative control wells, were encountered frequently. Troubleshooting was done to determine and eliminate the sources of contamination. Results from the agarose gel electrophoresis that was done, on some of these products from *TEF1α* gene PCR, are shown in **Figure 4.**

Following *in silico* analysis of the sequencing data, of the samples that amplified successfully during the *TEF1α* gene PCR, the presence of 11 different fungal genera were discovered. The successfully identified genera, based on the BLASTx results, consisted of both environmentally-abundant as well as some pathogenic fungal organisms and included *Botryosphaeria* spp., *Colletotrichum* spp., *Cylindrocarpon* spp., *Fusarium* spp., *Glomerella* spp., *Mucor* spp., *Neonectria* spp., *Ophiostoma* spp., *Pestalotiopsis* spp., *Phytophthora* spp. and *Trichoderma* spp.

Among the various fungal organisms that were identified from the *TEF1*α sequences was *F. euwallaceae.* This isolate's sequence, obtained from a backyard avocado tree,

in Sandton, in Johannesburg, was regarded as the first *F. euwallaceae* isolate obtained from avocado, in South Africa. This culture, stored in the CMW collection - FABI, University of Pretoria, will hereafter be referred to as CMW 51808.



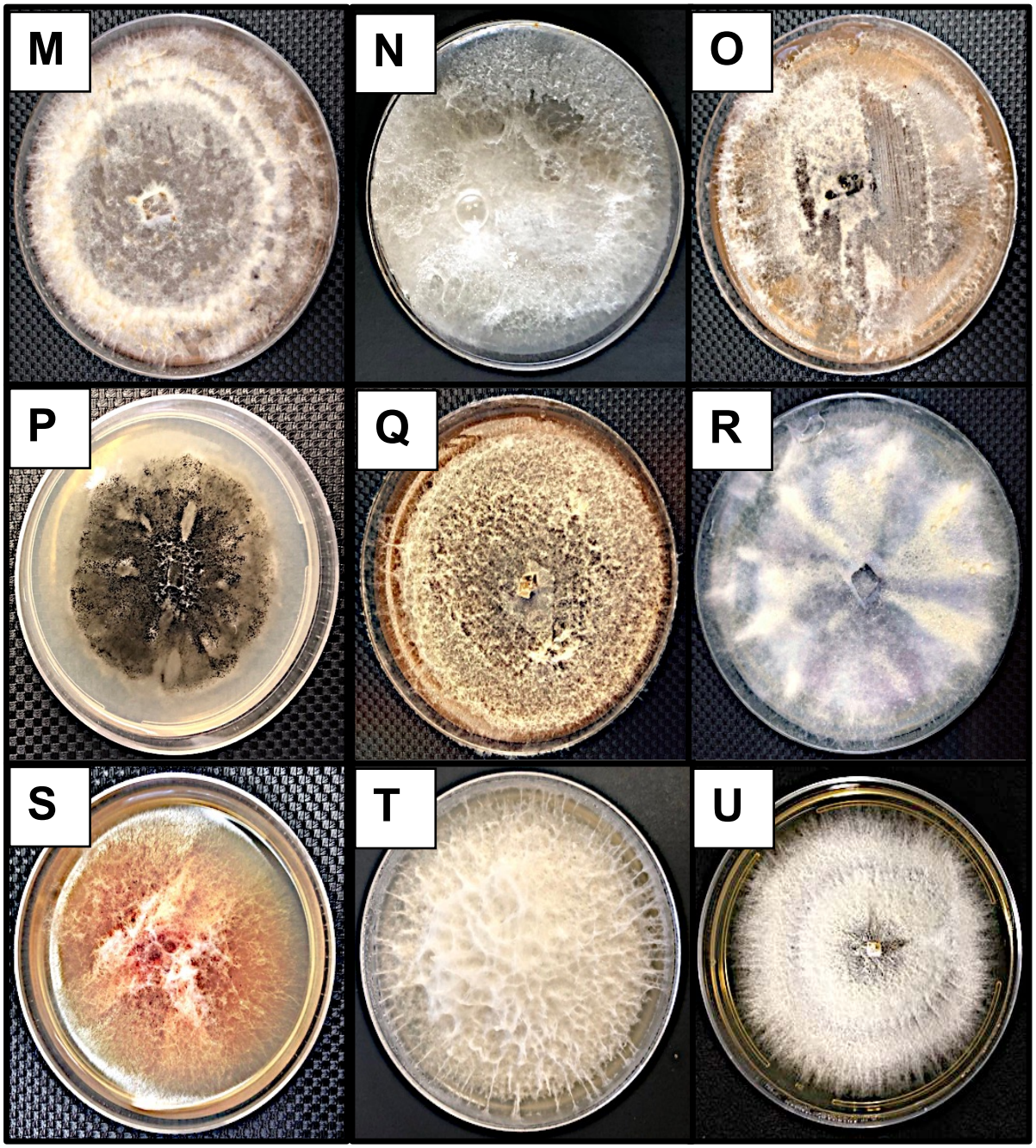


Figure 3: Representatives of the pure cultures that were obtained through primary isolations, performed on diseased avocado tissue. Some of the major groups identified through morphologic and molecular analysis included: *Fusarium* spp. (D, F, J, K, N, R, S, T), *Colletotrichum* spp. (C), *Botryosphaeria* spp. (L, P), *Mucor* spp. (H, Q), *Pestalotiopsis* spp. (A), *Trichoderma* spp. (O), *Neonectria* spp. (M), *Glomerella* spp. (E), *Cylindrocarpon* spp. (I, U), *Ophiostoma* spp. (G), and *Phytophthora* spp. (B).

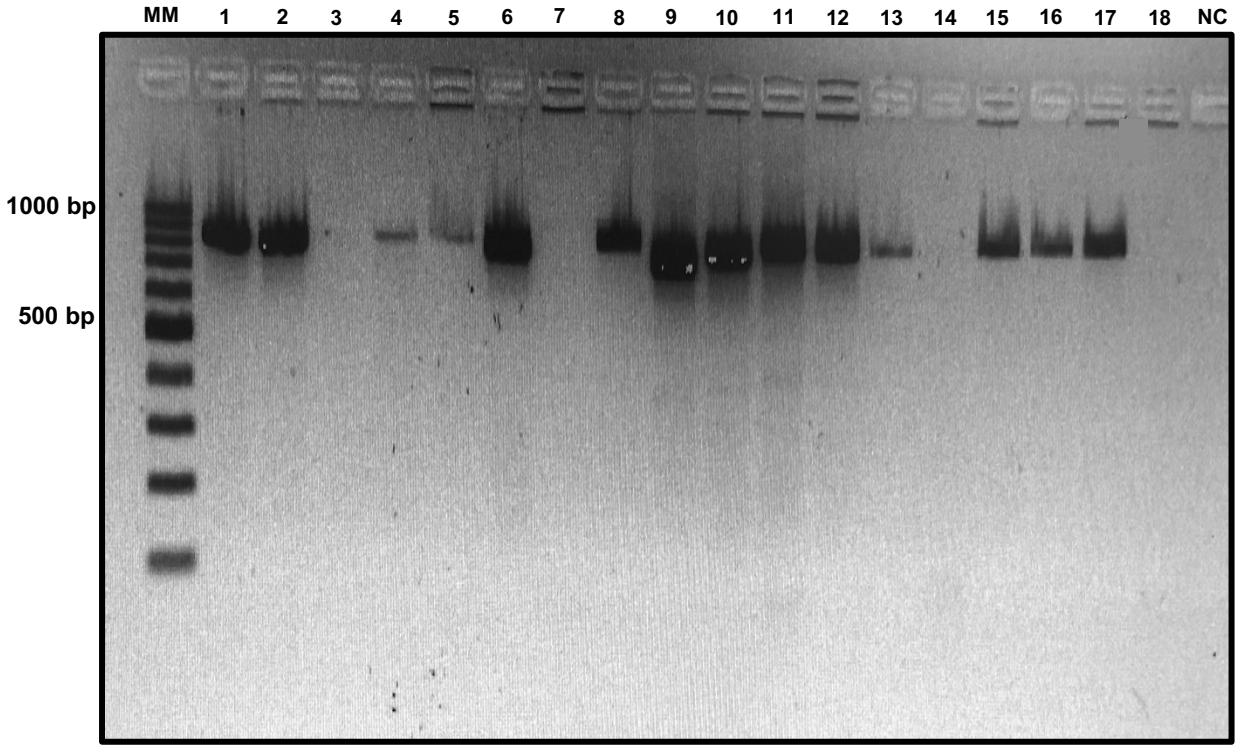


Figure 4: 2% agarose gel electrophoresis conducted on some of the products of the *TEF1α* gene PCR, performed on the fungal isolates DNA. All the samples for which successful amplification occurred, showed clear bands at around 700 bp – 800 bp. The lane labelled MM (Molecular Marker) indicates the banding pattern yielded from the Quick-Load 100 bp DNA ladder. The lack of any banding pattern at the lane labelled NC (Negative Control) indicates the absence of contamination. Samples represented by visible bands in these gels (1 – 2, 4 – 6, 8 – 13, 15 – 17) were retained for further studies and sequencing. The samples representing unamplified products (3, 7, 14, 18) were excluded from further studies.

Phylogenetic tree construction and identity inference of *Fusarium* spp.

A scarcity of depositions for the *TEF1α* gene region for *F. euwallaceae,* on the NCBI (National Center for Biotechnology Information) and other online fungal databases, meant that any sequences representing *F. euwallaceae* isolates were broadly classified as being part of the *Fusarium solani* species complex (FSSC), specifically *F. solani,* by the BLASTx analysis. The phylogenetic framework that resulted was used to confirm the exact species identity of the isolate (CMW 51808), as well as to determine whether other unknown cultures, from this study, defined as *Fusarium* spp., by the BLASTx analysis, may have been related to or shared identity with CMW 51808 or other ambrosial *Fusaria*. An example of the phylogenetic inference, used for accurately distinguishing *F. euwallaceae* isolates from the other members of the FSSC samples, obtained from this study, is indicated in **Figure 5.**

In addition to considering the sequence data from CMW 51808 contig, within an MSA, to determine its haplotype, the sequence was also studied phylogenetically, together with the sequences from the MSA, illustrated in **Figure 6**. This additional measure of accuracy provided further evidence to substantiate the identity and haplotype. This phylogenetic analysis was used to gain an overall perspective of the positioning of an isolate of interest, relative to other reported haplotypes for *F. euwallaceae* and ambrosial *Fusaria.* This study revealed that the partial *TEF1α* gene region of CMW 51808 shared an identical sequence with the *TEF1α* gene region of the *F. euwallaceae* isolates from avocado in Israel and California, proving identical haplotypes. This is of particular significance in order to evaluate the impact that has been associated with the presence of an invasive threat, in the areas where it has already successfully established.

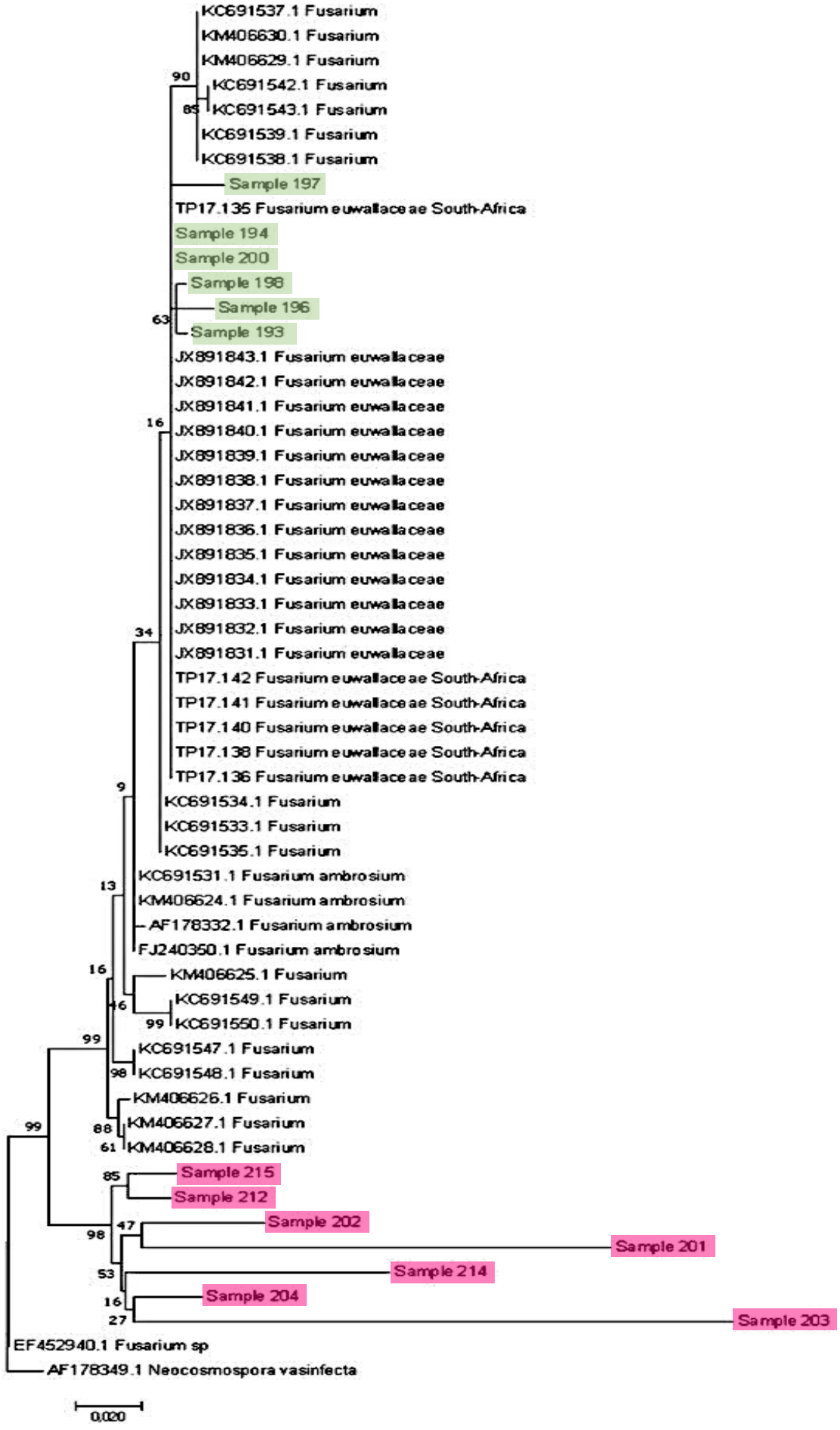
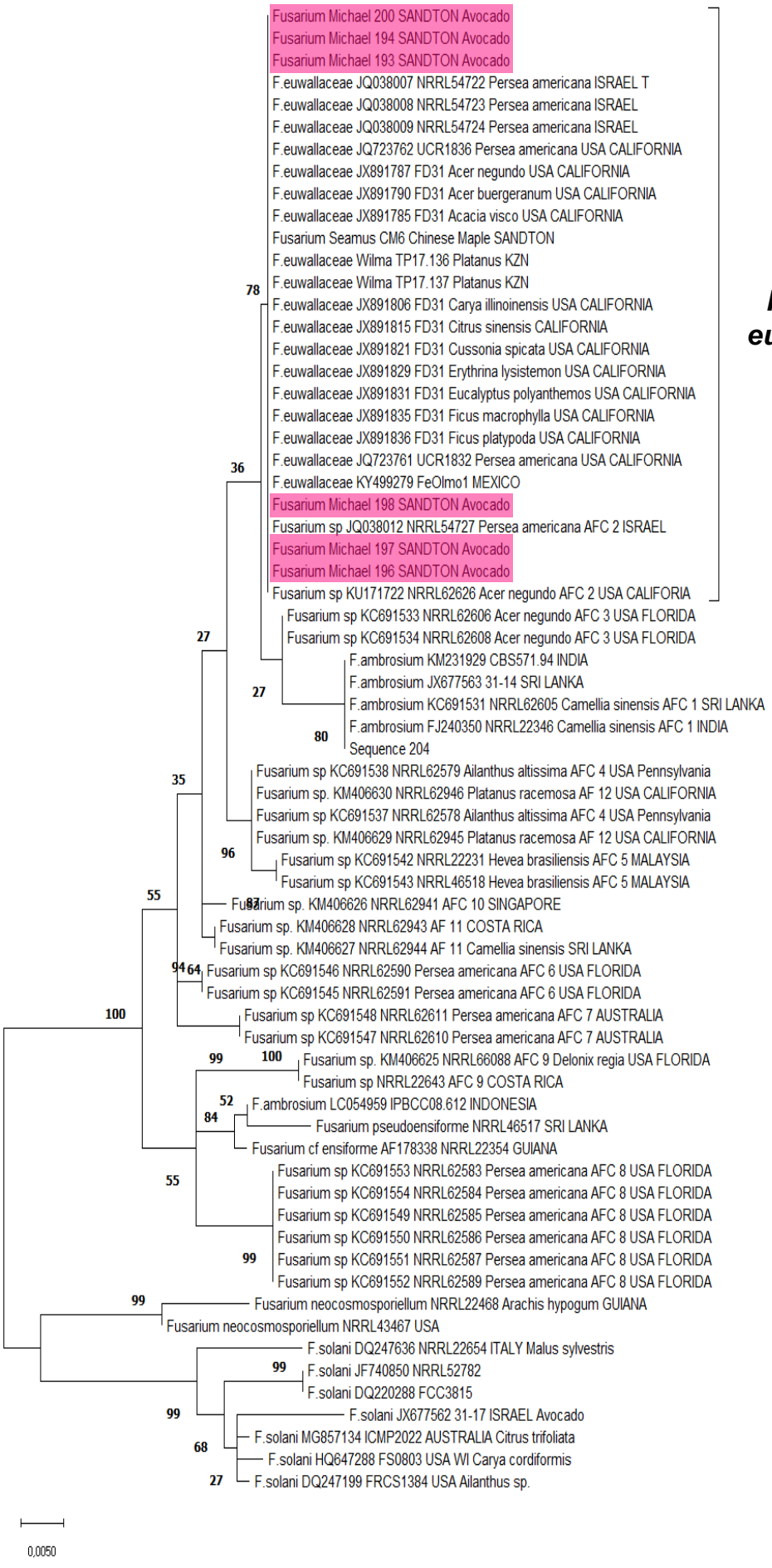

KC691537.1 Fusarium
KM406630.1 Fusarium
KM406629.1 Fusarium
KC691542.1 Fusarium
KC691543.1 Fusarium
KC691539.1 Fusarium
KC691538.1 Fusarium
Sample 197
TP17.135 Fusarium euwallaceae South-Africa
Sample 194
Sample 200
Sample 198
Sample 196
Sample 193
JX891843.1 Fusarium euwallaceae
JX891842.1 Fusarium euwallaceae
JX891841.1 Fusarium euwallaceae
JX891840.1 Fusarium euwallaceae
JX891839.1 Fusarium euwallaceae
JX891838.1 Fusarium euwallaceae
JX891837.1 Fusarium euwallaceae
JX891836.1 Fusarium euwallaceae
JX891835.1 Fusarium euwallaceae
JX891834.1 Fusarium euwallaceae
JX891833.1 Fusarium euwallaceae
JX891832.1 Fusarium euwallaceae
JX891831.1 Fusarium euwallaceae
TP17.142 Fusarium euwallaceae South-Africa
TP17.141 Fusarium euwallaceae South-Africa
TP17.140 Fusarium euwallaceae South-Africa
TP17.138 Fusarium euwallaceae South-Africa
TP17.136 Fusarium euwallaceae South-Africa
KC691534.1 Fusarium
KC691533.1 Fusarium
KC691535.1 Fusarium
KC691531.1 Fusarium ambrosium
KM406624.1 Fusarium ambrosium
AF178332.1 Fusarium ambrosium
FJ240350.1 Fusarium ambrosium
KM406625.1 Fusarium
KC691549.1 Fusarium
KC691550.1 Fusarium
KC691547.1 Fusarium
KC691548.1 Fusarium
KM406626.1 Fusarium
KM406627.1 Fusarium
KM406628.1 Fusarium
Sample 215
Sample 212
Sample 202
Sample 201
Sample 214
Sample 204
Sample 203
EF452940.1 Fusarium sp
AF178349.1 Neocosmospora vasinfecta
0.020

Figure 5: An example of the maximum likelihood phylogenetic tree generated, based on the *TEF1α* gene region, used to identify the unknown isolates and to distinguish *Fusarium euwallaceae* isolates from the other members of the *Fusarium solani* species complex (FSSC). Within this phylogenetic tree, samples indicated as: 193, 194, 196, 197, 198 and 200 (highlighted in green) represent replicates sequenced from the same isolate – namely CMW 51808. It is clear how this isolate phylogenetically grouped/associated with other selected *F. euwallaceae* sequences, meaning that its identity can be phylogenetically inferred to be *F. euwallaceae*. In contrast the remaining sequences, labelled as 201, 202, 203, 204, 212, 214, and 215 (highlighted in pink) form a separate branching point and cluster far away from *F. euwallaceae* meaning that they are within the genus of *Fusarium,* but they are unlikely to be part of the FSSC and far less likely to be *F. euwallaceae.*



Fusarium euwallaceae

Figure 6: A phylogenetic tree inferred from partial *TEF1*α sequences using Maximum Likelihood, in MEGA7. *Fusarium euwallaceae* isolates (replicates of CMW 51808) from avocado, in South Africa (highlighted in pink) share identical sequences with those from avocado in Israel and California, proving identical haplotypes. Values obtained from 1000 bootstraps are identified on internodes.

Koch's postulates to confirm *Fusarium euwallaceae* (isolate CMW 51808) as the causal agent for the disease on avocado

The successfully identified isolate of *F. euwallaceae* (CMW 51808) was used to conduct a pathogenicity test, towards fulfilling Koch's postulates and concluding that it was the causal agent for the disease symptoms that were observed.

Following incubation, external symptoms of disease, observed in the pathogen-inoculated plantlets included wilting and discoloration and drooping of the foliage. Plentiful sugary exudates were also observed around the parafilm, at the artificially-wounded inoculated sites. The internal symptoms included clear reddish-brown pigmentation in the vascular tissue and water-soaked lesions, with an average measurement of 4.56 cm. The results for this pathogenicity trial are indicated in **Figure 7** (control plants free of visible disease symptoms) and **Figure 8** (infected plants that show symptoms of FD). The lesion lengths and quantitative data from the pathogenicity trial are indicated in **Table 2**, in the supplementary information**.** The statistical analysis thereof showed that the data followed Gaussian distribution and a one-way-ANOVA proved that the average lesions lengths observed for the inoculated plants differed significantly from the average lesions lengths observed for the control plants. This data is illustrated, in **Figure 9.**

Primary isolations from the wounds, aimed at isolating septate, clavate macroconidia with slight dorsoventral curvatures, swollen in their upper halves, from the lesions, successfully re-isolated *F. euwallaceae* from the inoculated plants and not from the control plants. The successful infection, re-isolation from the artificially inoculated plants, presenting symptoms of FD, and confirmation of identity by sequencing the *TEF1α,* β-tubulin and *RBP2* gene regions fulfilled Koch's postulates and concluded that *F. euwallaceae* was the cause of the disease.

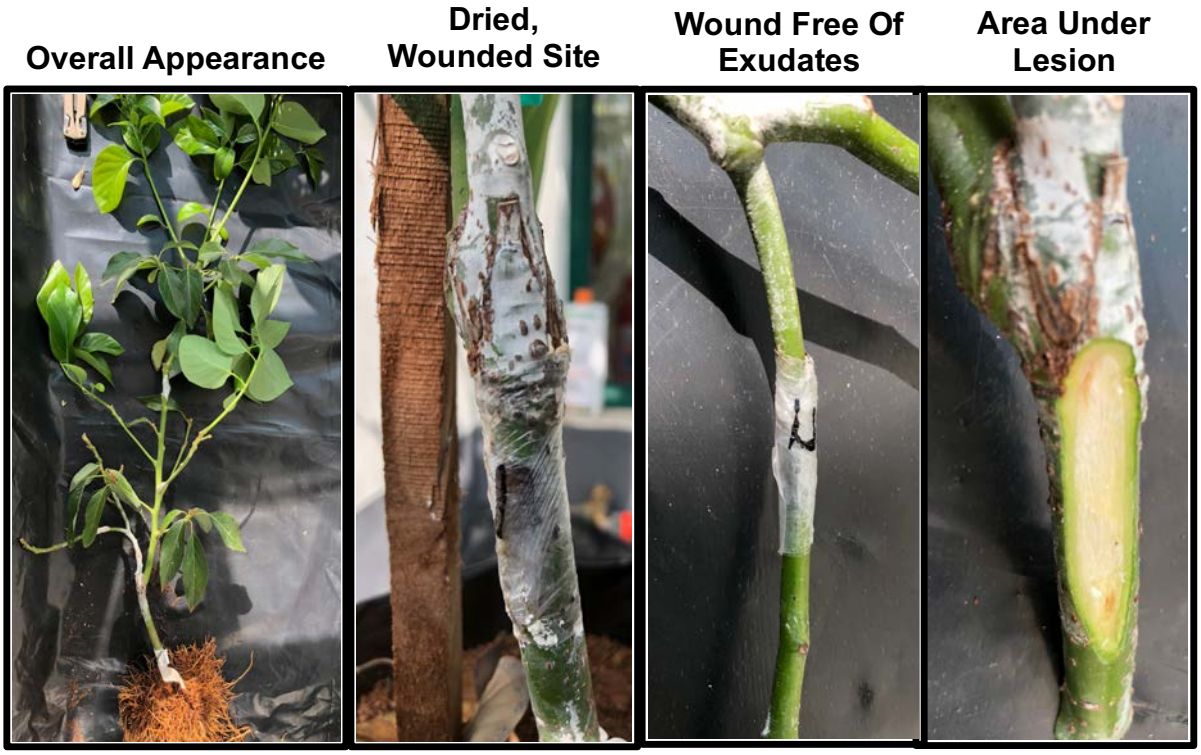


Figure 7: The control plants that were inoculated with sterile, ½ PDA plugs during the pathogenicity trial. After incubation, the artificial wounds made on the control plants were dried out and completely free of sugary exudates. Upon removal of the parafilm, the lesions were observed as being dry and stripping away of the plant tissue around the wounds showed a clear absence of pathogenic infection. The leaves on the control plants were also much more plentiful and noticeably healthier – particularly given the appearance of new leaves and an overall brighter green colour. Primary isolations were performed from the wounded sites and no *Fusarium euwallaceae* was isolated.

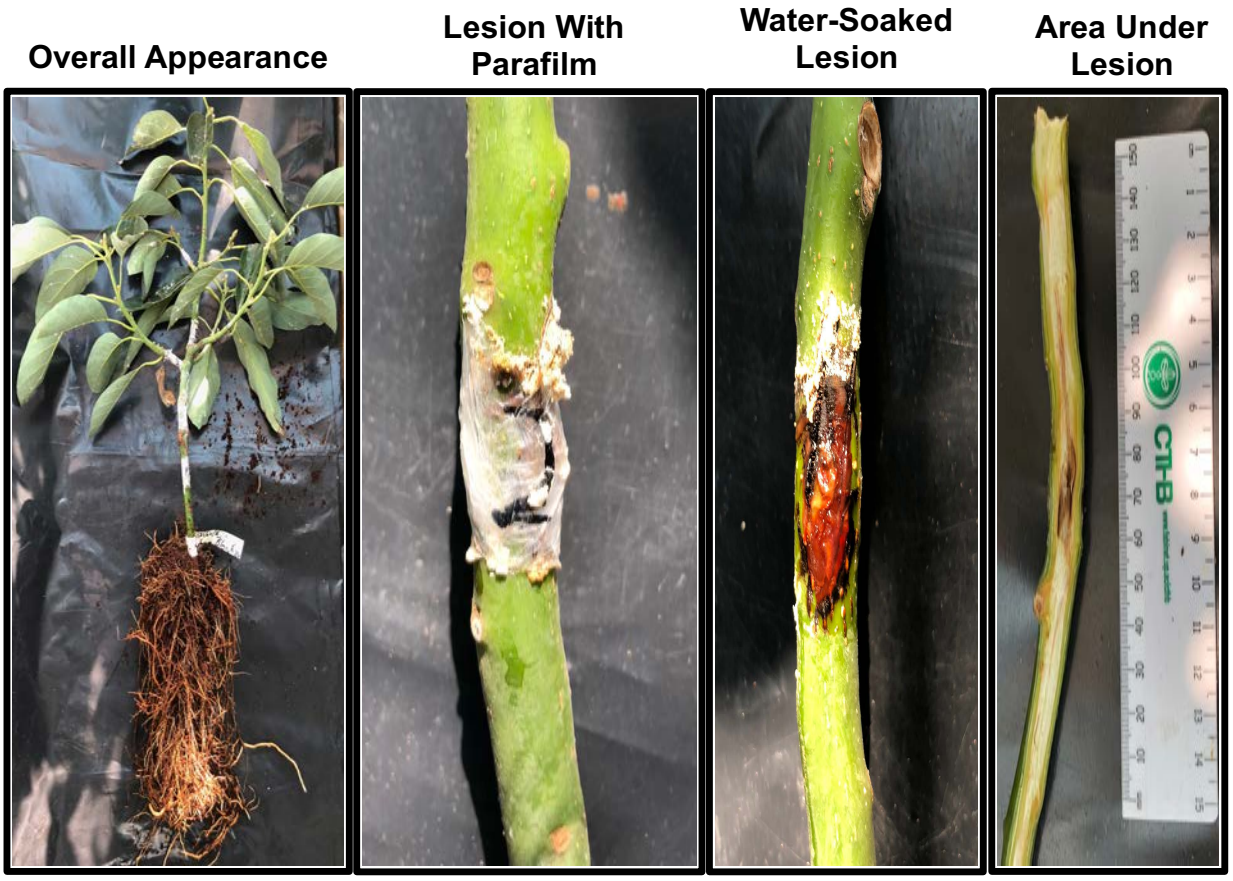


Figure 8: The plants inoculated with *Fusarium euwallaceae* (CMW 51808) during the pathogenicity trial. The lesions marked the presence of expected sugary exudates, associated with Fusarium dieback, on avocado. Plentiful sugar exudates surrounded the border of the parafilm, in virtually every wound on the infected plants. Upon removal of the parafilm, a water-soaked lesion was observed. To measure the actual length of the lesion, as well as the degree of spread into the vascular tissue, some tissue, on either side of the lesion, was stripped away, revealing an extensive spread of the pathogen. Most of the leaves, on almost all of the infected samples, had dropped or were drooped, presenting a dull green colour – accounted to a decrease in the efficiency of the water conducting vascular tissue. Primary isolations were performed from the lesions, thereby successfully re-isolating *F. euwallaceae.*

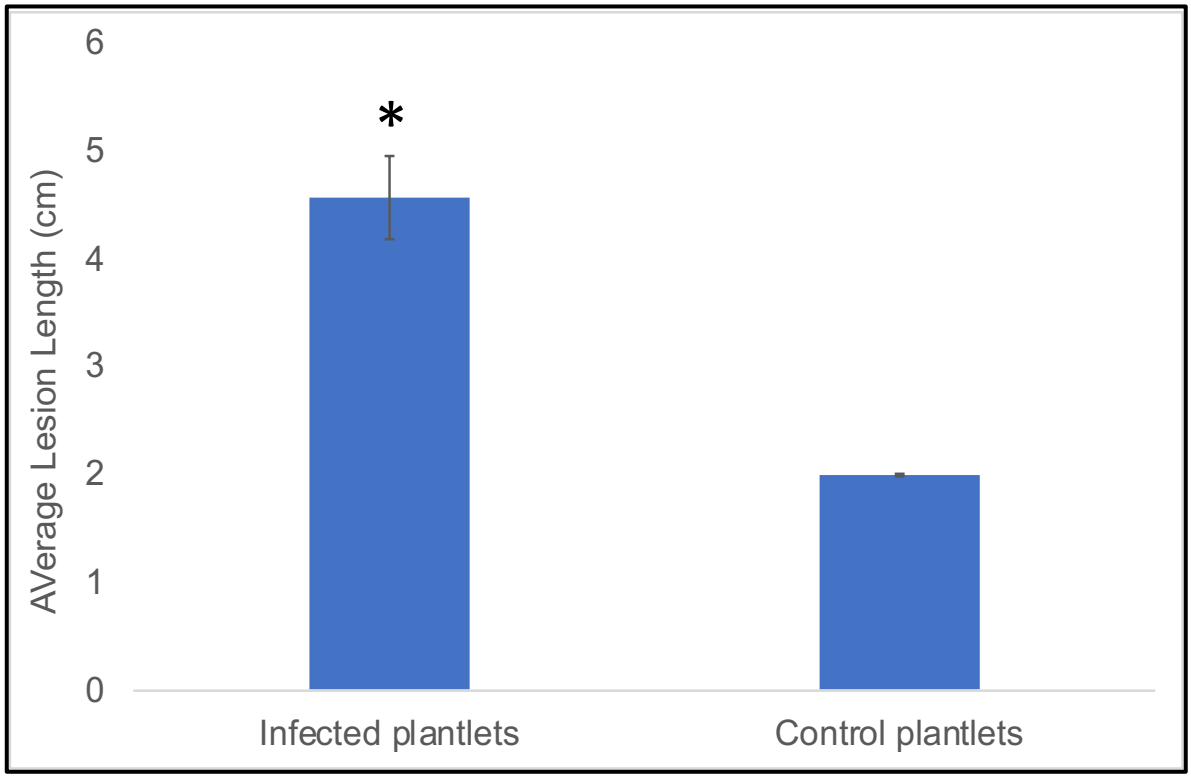


Figure 9: A column bar graph, representing the data set generated by the measurements of average lesion lengths caused by *Fusarium euwallaceae*, during the pathogenicity trial. The statistical analysis revealed normal distribution of the data and a significant difference between the mean lesions lengths of the infected plants compared to the control plants, indicated with the asterisk. Standard error for both groups are also indicated, as a measure of the amount of deviation from the mean, for each data set.

Identification of beetles from avocado orchards

Unknown beetles were studied by considering diagnostic features observed for the PSHB, including the appearance of the head shape and size, pronotum width and length, antennae, conspicuous features such as serrations or hairs on the head and elytron, punctures on the lateral margin, leg shape and size, elytral length and width and degree of elytral declivity.

Some of the key, morphological characteristics that were used to distinguish non-ambrosia beetles and other ambrosia beetles from the PSHB, are indicated by the images taken of the various planar angles, of a confirmed PSHB specimen, in **Figure 10.** The features observed included a lack of conspicuous surface features, except for hairs that are arranged in rows across the entire elytron. The surface of the declivity was unarmed by projections and is only covered with strial punctures and interstrial tubercles, granules and hairs. The PSHB is brown-black and has a tall and broadened pronotum. The colour of the observed beetle was uniform with a size of about 1.8 to 2.5 mm. The anterior margin of the pronotum was also smooth and clearly devoid of serrations. The PSHB has a gently descending elytron that is never drastically sloped, indented or truncated and the elytron is about 1.5x longer than the head and pronotum.

The beetles collected and related to disease on avocado were broadly separated based on a list of key morphological characteristics, using light microscopy and major beetle families and genera were identified, where possible. The main beetle sub-family identified was the Scolytinae, with some other specimens from Bostrichidae and Platypodinae. Many ambrosia beetles from the Xyleborini tribe were discovered and some of the major genera that were morphologically identified include *Xylosandrus* spp., *Xylion* spp., *Xyleborinus* spp., *Xyleborus* spp., *Premnobius* spp., *Sinoxylon* spp.,

Euwallacea spp., *Enneadesmus* spp., *Eccoptopterus* spp., *Scolytoplatypus* spp., *Thamnurgus* spp., *Xyloperthodes* spp., *Diuncus* spp., *Ambrosiodmus* spp. and *Hylastes* spp. Microscopic imaging, for some of these beetles, is shown in **Figure 11.** The sampling also revealed significant differences in the abundances of the various beetles. Members from the *Xylosandrus* genus were by far the most plentiful, in all three provinces that were sampled, and represented approximately 33% of the total amount of beetles that were collected in this study. The second most abundant was the *Premnobius* genus and represented 19% of the total amount of beetles that were collected. The remaining beetle genera were detected in relatively low numbers, however, it is noteworthy that almost all of the *Premnobius* spp. and the majority of the other beetles were acquired from avocado orchards in Tzaneen, Limpopo.

Results from the agarose gel electrophoresis, of the PCR products for the *COX 1* gene region, indicated persistently failed amplification. Subsequent analysis proved that all the DNA extraction reactions were unsuccessful, meaning that identification and separation of the beetles in this study relied on the use of morphological techniques alone.

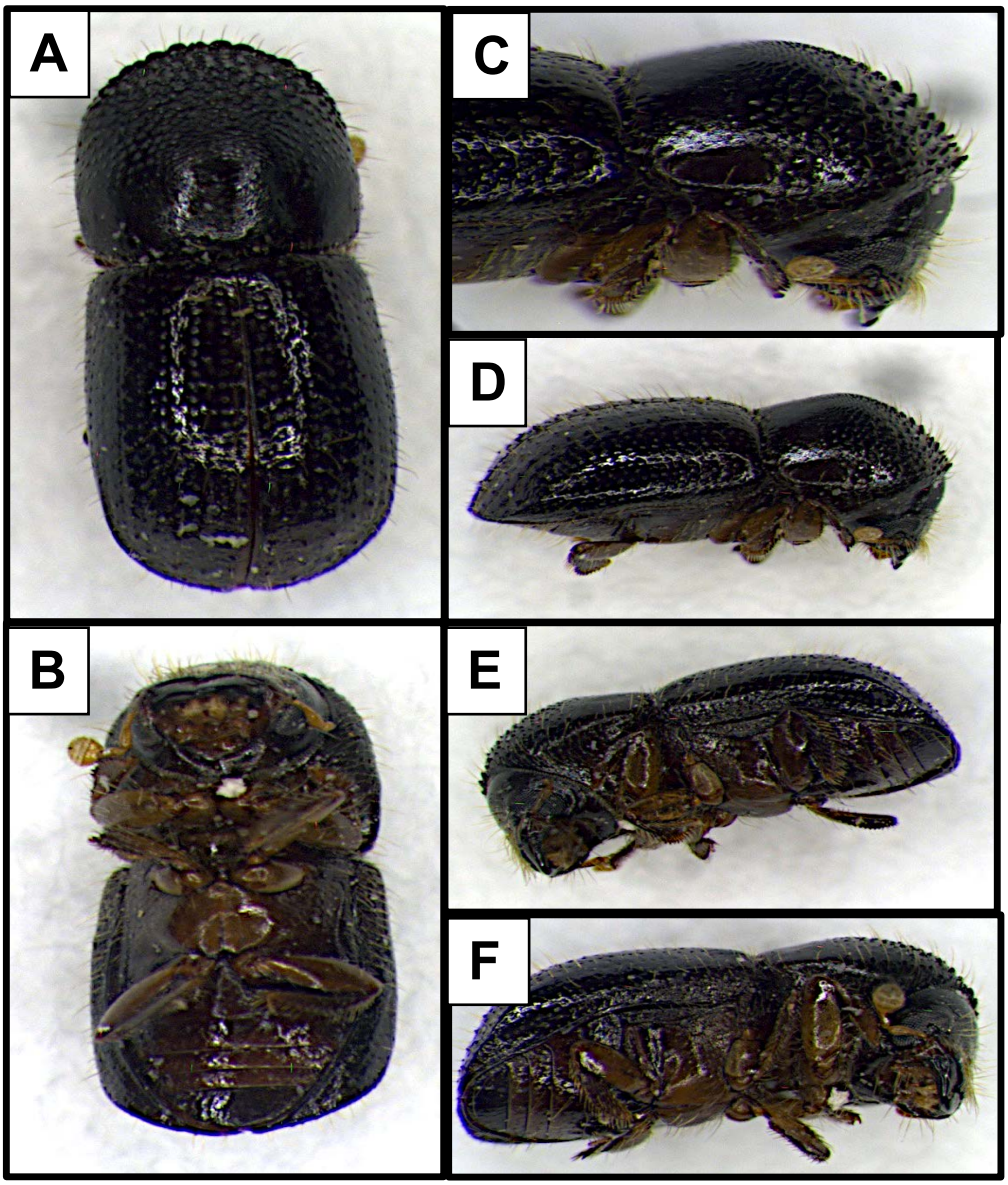
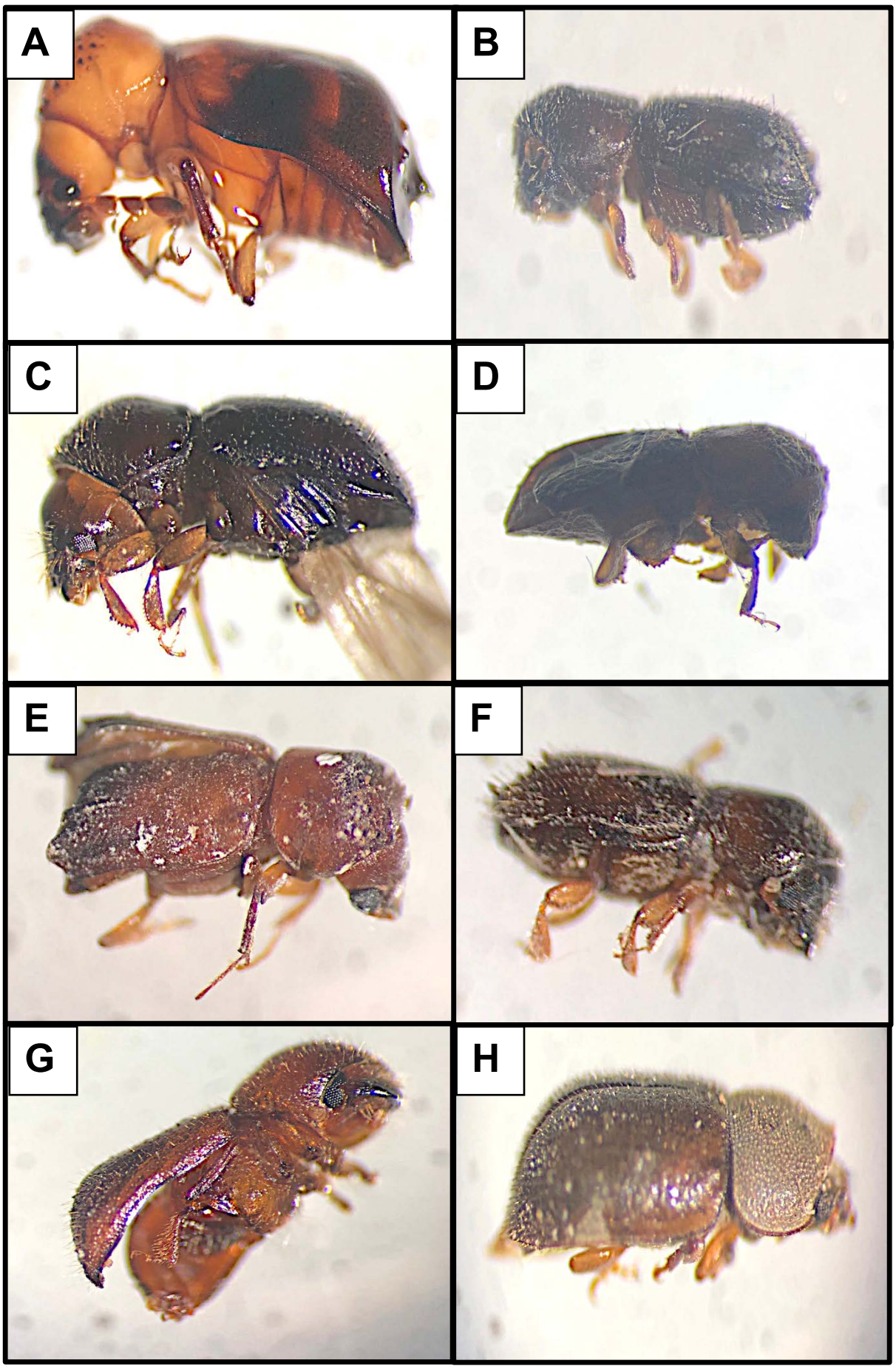


Figure 10: High-resolution photographs taken from various angles, of a confirmed polyphagous shot hole borer (PSHB) ambrosia beetle, used as the reference organism for the comparison of unknown beetles. **A –** Dorsal view. **B –** Ventral view. **C –** Anterior cranial view. **D –** Lateral view. **E – F:** Inferior and superior, lateral views. The images indicate a lack of conspicuous surface features, except for hairs that are arranged in rows across the elytron. Also indicated, in the above, is the correct colour and size relationship between the head and elytron. Together with the other observations, a gently descending elytral declivity indicate the morphological characteristics of the PSHB.


A
B
C
D
E
F
G
H

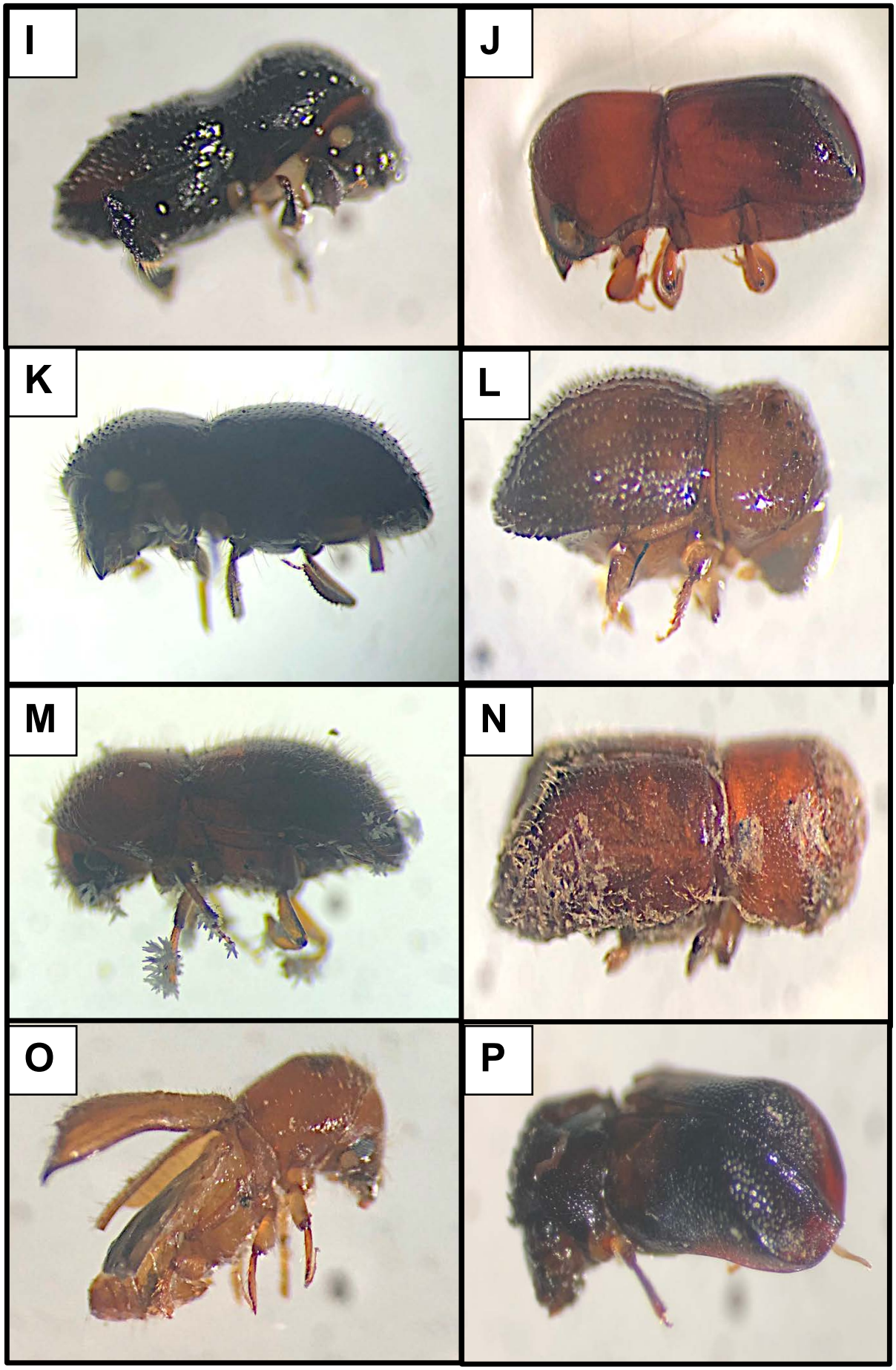

I
J
K
L
M
N
O
P

Figure 11: The morphology of the major groups of beetles collected and captured from avocado tissue and lesions. All beetles indicated from: **A – P** were deemed not to be the polyphagous shot hole borer (PSHB), due to morphological characteristics alone. These characteristics are showcased in **A – P** and indicate: Incongruent overall colour and total size of the beetle as well as its features, such as the pronotum, head, legs and elytron. In many cases the principle means for concluding dissimilarity from the PSHB was due to the presence of major conspicuous surface features, such as spines, barbs or jagged projections. Many beetle samples that were surveyed also displayed prominently armed elytra and/or sharply descended elytral declivities.

DISCUSSION

The main and most significant finding of this study was the detection of the PSHB (*E. fornicatus*) and *F. euwallaceae* causing disease on avocado, in South Africa, for the first time (van den Berg *et al.*, 2019) – publication accessed on: doi.org/10.1094/PDIS-10-18-1818-PDN. This pest complex has caused extensive damage to a variety of hosts in Israel and California, in the United States of America, specifically on avocado (Eskalen *et al.*, 2012, Mendel *et al.*, 2012, Umeda *et al.*, 2016). This study shared a similarity with that of the first detection in California, in that it was detected on a backyard avocado tree, for the first time (Eskalen *et al.*, 2012). Subsequent genetic and phylogenetic analysis of the *F. euwallaceae* isolate, obtained from this study, verified that the universally conserved translation elongation factor 1α (*TEF1*α) gene region of the South African isolate, from avocado, shared identical DNA sequences with the *F. euwallaceae* isolates from avocado in Israel and California. The pathogenicity trial and phylogenetic inferences proved that a single, internationally invasive haplotype of this fungus is spreading globally and is responsible for the

damage observed locally, in Israel and the USA. Extrapolations of the potential impact, in South Africa, can therefore be gained from the other countries, where this pest has already established.

In California, the PSHB has spread despite the application of control means and is considered a serious threat to local plant-based biodiversity (O' Donnell *et al.*, 2016). Native and ornamental trees, forests and shrubs are considered to be under critical threat by the PSHB pest complex, in California (Tyler *et al.*, 2019). However, from an agricultural perspective, particularly the avocado growing industry, this pest is considered of lesser impact, compared to some of the other pest species that have far more devastating effects and is well managed through the pruning and destruction of infested plant material (O' Donnell *et al.*, 2016). In the United States of America, the threat posed by another ambrosia beetle and its fungal symbiont – the Redbay ambrosia beetle and *Raffaelea lauricola* T.C. Harr., Fraedrich & Aghayeva, causing Laurel wilt*,* seriously threatens not only avocado (Ploetz *et al.*, 2012), but all members of the *Lauraceae* genus (Kendra *et al.*, 2013). In contrast to the dieback symptoms and semi-localised infection, that can be managed by early pruning and destruction of the infested material, observed by plants affected with FD, Laurel wilt causes a systemic infection and is regularly lethal to infested trees (Kendra *et al.*, 2013), despite pruning and removing infested plant material. This threat currently outshines the threat posed by the PSHB pest complex and FD, in the USA, however further geographic spread may prove to infer serious economic and biodiversity threats.

In Israel, the contrary is observed, in that this pest complex has been associated with major repercussions to agriculture by incrementally debilitating the avocado growing industry, as its spread has progressed. In Israel, the threat posed by the PSHB was

exacerbated by unequipped management practices, similar to what is observed in South Africa, thereby establishing a country-wide presence within a couple of years (Carrillo *et al.*, 2016). The PSHB is currently present in the majority of avocado cultivation areas and threatens the long-term viability, sustainability and feasibility of avocado growing, within Israel (Mendel *et al.*, 2012).

In a developing country like South Africa, the introduction of this pest could impact agriculture and the avocado industry, similarly to Israel, if management practices are not sufficiently implemented, to manage infested material. However, the more apparent threat to South Africa lies in the country's abundance of plant life and biodiversity. South Africa is known as a major contributor to global ecological plant diversity and is home to more than 22 000 indigenous seed plants, from almost 230 families (Mucina & Rutherford, 2006). The polyphagous nature and broad host-range of *E. fornicatus* has the potential to warp and alter the structure of local environments and ecosystems, particularly in areas that are not routinely monitored (Ploetz *et al.*, 2013). This may result in losses to indigenous biodiversity as well as prompting the succession of an affected area with alien plant species. In addition, a general lack of attention, to ambrosia beetle damage and associated control strategies, in South Africa, means that local communities are unaccustomed to dealing with a threat that can spread completely autonomously, thereby increasing the potential risk of spread.

The recent detection of the PSHB and its accompanying fungal symbionts, on a broad range of hosts, in South Africa, highlights the necessity for far more stringent invasion biology studies and local border security controls. In this case, the arrival and establishment of this pest complex has been detected relatively early (Paap *et al.*, 2018, van den Berg *et al.*, 2019), meaning that the monitoring and application of basic

and applied research will act as a major deciding factor, to determine the spread and associated damage by this pest complex, in the future.

During the monitoring and surveying conducted in this study, in addition to detecting the PSHB and its fungal symbiont (*F. euwallaceae)*, several other ambrosia beetles and their accompanying fungi were detected. When performing primary isolations from plant tissue, suspected to be affected by FD, the first means of detection relies on culturing and morphologically screening and selecting suspicious fungal organisms. However, the growth generated by numerous other, naturally-occurring micro-organisms can result in difficulties to achieve pure cultures, but more importantly to correctly select the organism of interest. When initially cultured onto nutrient media *F. euwallaceae* shares countless morphological similarities with many naturally occurring fungi, thereby drastically increasing the difficulty of detecting its presence from conventional primary isolations. It was therefore essential that thorough sub-culturing was performed from each primary isolation.

As a result, several epiphytic, pathogenic and environmentally-occurring fungal genera were detected from the isolation of diseased avocado tissue, by means of morphologic and molecular techniques. These fungal genera including *Botryosphaeria* spp., *Colletotrichum* spp., *Cylindrocarpon* spp., *Glomerella* spp., *Mucor* spp., *Neonectria* spp., *Ophiostoma* spp., *Pestalotiopsis* spp., *Phytophthora* spp. and *Trichoderma* spp. Persistent isolations from the diseased avocado tissue also yielded various members of the *Fusarium* genus, including *F. euwallaceae*. Subsequent genetic and phylogenetic analysis of the other isolates revealed an abundance of *Fusarium solani* and *Fusarium oxysporum.* Many of the symptomatic avocado trees that were sampled and isolated from, and proved to be free of the PSHB, allowed for

the isolation of *F. solani.* The continuous isolation of *F. solani* from diseased avocado trees, damaged by wood-boring beetles, is potentially troublesome. Many of the members of the FSSC are ambrosial fungi and are often considered as ubiquitous agricultural pathogens, on their own (Coleman, 2016). Members of the FSSC are also known to undergo host shifts (O'Donnell *et al.*, 2015) and although unlikely may develop chance symbioses with normally un-invasive ambrosia beetles (Hulcr & Stelinski, 2017). Therefore, if these pathogenic members, of the FSSC, are consistently associated with- and isolated from diseased avocado tissue in the future, the continued association with diseased avocado needs to be critically researched and evaluated.

Collecting beetles from symptomatic avocado trees, suspected to have sustained beetle damaged, yielded a large variety of beetles, specifically members of the Scolytinae subfamily. The majority of DNA extractions conducted on these beetles were unsuccessful, as many beetle samples that were received were not effectively preserved, not preserved in a sufficient amount of time or not preserved at all. In addition, since most DNA extraction protocols rely on the use of sufficient test material i.e. entire beetle specimens, in an attempt to yield sufficient DNA concentrations, each failed attempt at extraction meant the permanent loss of the beetle specimen. Therefore, after successive failures to extract DNA, the main detection technique used to eliminate non-PSHB beetles, was comparative light microscopy. This process involved observing unknown beetles alongside a confirmed sample of the PSHB ambrosia beetles and paralleling their features to determine whether the unknown beetles had any morphological overlap, with the key, morphologic PSHB features.

The use of such morphologic approaches alone has its obvious limitations. Closer inspection of bark and ambrosia beetle taxonomy and morphology reveals the same conundrum that is shared with the identification of micro-organisms, in that many different, yet related, beetle species can share almost identical morphologies (Bright & Stark, 1973). The implication thereof is that morphological approaches, to identify unknown beetle specimens, are supported by far less scientific surety, particularly in the light of the effectiveness and certainty that is displayed when using molecular approaches. This leads to the possibility of false-positive identifications of the incorrect beetle species. Despite the numerous extraction protocols applied during this study, there is a requirement for a more tailored DNA extraction protocol, specific to the PSHB. Such an optimised extraction protocol would be aimed at increasing the chances of yielding successfully-extracted, high-quality DNA, as well as the prospect of requiring less test material, in an attempt to preserve the key morphological features of the beetle being studied.

Nonetheless, morphological considerations were used as the only identification technique and thereby detected several members of the Xyleborini tribe. Several genera from the Xyleborini tribe included *Xylosandrus* spp., *Xyleborinus* spp., *Xyleborus* spp., *Eccoptopterus* spp., *Diuncus* spp. and *Ambrosiodmus* spp. This observation is of particular interest, as some of the ambrosia beetles within the Xyleborini tribe are notorious invasive species (Hulcr *et al.*, 2007). Damage associated with these ambrosia beetles is usually restricted to young trees and nursery stock, but these beetles have also been observed to attack apparently healthy or stressed trees (Kirkendall *et al.*, 2015). Despite the numerous detections of these ambrosia beetles, they have not traditionally been considered as serious pests in South Africa, or pests on healthy, mature avocado trees. However, less conspicuous and even unassuming

ambrosia beetles from abroad should be considered as potential concerns as they may become significant pests once established in the new environment and climate (Haack, 2006), afforded by South Africa. Evidence from this study, related to the abundance as well as consistent association of these otherwise unassuming beetles, with damage to stressed and healthy avocado trees, suggests the possibility of an emerging threat.

To date, despite continuous surveying, there has been no detection of the PSHB pest complex in the major commercial avocado growing areas, in South Africa. However, the autonomous movement, through the flight of female beetles, means that the spread will likely continue and will cause damage, to not only avocado but to various other reproductive and non-reproductive plant species. In the light hereof, it is essential that the early detection, reported on in this study, be considered as a serious warning sign. This should prompt the active monitoring of this invasive threat and ensure that control measures are devised accordingly. Furthermore, proper management practices of infested plant material should be put in place to combat and lessen the effect posed by this beetle and its accompanying pathogenic partner.

The aims of this study were achieved by successfully raising awareness of the presence of this newly introduced pest. Together with other projects, initiated to study this disease, this research contributed to the overall knowledge pool regarding this invasive pest, specifically on avocado. In addition, by utilising and testing the currently available morphologic, molecular and phylogenetic detection approaches, the essential tools for the future monitoring of this and other beetle-fungus complexes were highlighted.

Due to the nature of the first-hand encounter with these ambrosia beetles and their accompanying symbionts, many difficulties and challenges were encountered with regards to effective, accurate and time-efficient processing of samples and data. An overall scarcity of local and international publications, specific to the pathogen and its vector, meant that a large portion of the experimental work, included in this and the next study, was based on trial-and-error and resulted in many inconclusive results. Even so, this research, protocols and findings of this study summarise the tools and provide a foundation that can be used for further surveillance of the prevalence of FD, on avocado and other hosts, in South Africa.

CONCLUSION

The recent detection of the PSHB beetle-fungus pest, in South Africa, is of major concern from an economic and biodiversity standpoint. An international perspective of the damage associated with newly introduced pathogen-vectoring pests, such as ambrosia beetles indicates potential “black-swan effects”, for far-reaching and unpredictable effects (Ploetz *et al.*, 2013). Ongoing global studies regarding the invasion of ambrosia beetles have proven that the avocado growing industry will be one of the most directly and severely affected. Given the magnitude of the local avocado growing industry, the value of propagated, pathogen-resistant, plant material and the overall global contribution for the total export of avocado fruit, from South Africa, it is essential that this study and the discovery of the PSHB be considered as a threat of pressing importance.

The impact of the research generated by this study provides supposition towards the importance of acquiring funding, interest and research, to perform further studies on avocado as well as the other, native and indigenous hosts that may be affected. By

using the pipeline and detection approaches, followed by this study, an important foundation was lain towards understanding the basic aspects of the disease lifestyle, as well as determining the geographical spread of this pest, in South African orchards. The contribution of this basic research adds to the consideration of the overall health of the avocado fruit crop, meaning that the impact of such pathogen emergences should be extensively investigated, to assess the potential future threats posed to the avocado growing industry.

REFERENCES:

Aljanabi SM & Martinez I (1997) Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Research* **25**: 4692-4693.

Bright DE & Stark RW (1973) The bark and ambrosia beetles of California (Coleoptera: Scolytidae and Platypodidae). *The bark and ambrosia beetles of California (Coleoptera: Scolytidae and Platypodidae)* **16**.

Carrillo D, Cruz L, Kendra P, Narvaez T, Montgomery W, Monterroso A, De Grave C & Cooperband M (2016) Distribution, pest status and fungal associates of *Euwallacea* nr. *fornicatus* in Florida avocado groves. *Insects* **7**: 55.

Coleman JJ (2016) The *Fusarium solani* species complex: ubiquitous pathogens of agricultural importance. *Molecular Plant Pathology* **17**: 146-158.

DiGuistini S, Wang Y, Liao NY, Taylor G, Tanguay P, Feau N, Henrissat B, Chan SK, Hesse-Orce U & Alamouti SM (2011) Genome and transcriptome analyses of the mountain pine beetle-fungal symbiont *Grosmannia clavigera*, a lodgepole pine pathogen. *Proceedings of the National Academy of Sciences* **108**: 2504-2509.

Einax E & Voigt K (2003) Oligonucleotide primers for the universal amplification of *β-tubulin* genes facilitate phylogenetic analyses in the regnum Fungi. *Organisms Diversity & Evolution* **3**: 185-194.

Eskalen A, Gonzalez A, Wang D, Twizeyimana M, Mayorquin J & Lynch S (2012) First report of a *Fusarium* sp. and its vector tea shot hole borer (*Euwallacea fornicatus*) causing Fusarium dieback on avocado in California. *Plant Disease* **96**: 1070-1070.

Eskalen A, Stouthamer R, Lynch SC, Rugman-Jones PF, Twizeyimana M, Gonzalez A & Thibault T (2013) Host range of Fusarium dieback and its ambrosia beetle (Coleoptera: Scolytinae) vector in southern California. *Plant Disease* **97**: 938-951.

Freeman S, Sharon M, Maymon M, Mendel Z, Protasov A, Aoki T, Eskalen A & O'Donnell K (2013) *Fusarium euwallaceae* sp. nov.—a symbiotic fungus of *Euwallacea* sp., an invasive ambrosia beetle in Israel and California. *Mycologia* **105**: 1595-1606.

Haack RA (2006) Exotic bark-and wood-boring Coleoptera in the United States: recent establishments and interceptions. *Canadian Journal of Forest Research* **36**: 269-288.

Hulcr J & Stelinski LL (2017) The ambrosia symbiosis: from evolutionary ecology to practical management. *Annual Review of Entomology* **62**.

Hulcr J, Dole SA, Beaver RA & Cognato AI (2007) Cladistic review of generic taxonomic characters in Xyleborina (Coleoptera: Curculionidae: Scolytinae). *Systematic Entomology* **32**: 568-584.

Karlsson I, Edel-Hermann V, Gautheron N, Durling MB, Kolseth A-K, Steinberg C, Persson P & Friberg H (2016) Genus-specific primers for study of Fusarium communities in field samples. *Applied Environmental Microbiology* **82**: 491-501.

Kendra PE, Montgomery WS, Niogret J & Epsky ND (2013) An uncertain future for American Lauraceae: a lethal threat from redbay ambrosia beetle and laurel wilt disease (a review). *American Journal of Plant Sciences* **4**: 727.

Kirkendall LR, Biedermann PH & Jordal BH (2015) Evolution and diversity of bark and ambrosia beetles. *Bark Beetles:* 85-156. Elsevier.

Kolar CS & Lodge DM (2001) Progress in invasion biology: predicting invaders. *Trends in Ecology & Evolution* **16**: 199-204.

Liu YJ, Whelen S & Hall BD (1999) Phylogenetic relationships among ascomycetes: evidence from an RNA polymerse II subunit. *Molecular Biology and Evolution* **16**: 1799-1808.

Mendel Z, Protasov A, Sharon M, Zveibil A, Yehuda SB, O'Donnell K, Rabaglia R, Wysoki M & Freeman S (2012) An Asian ambrosia beetle *Euwallacea fornicatus* and its novel symbiotic fungus *Fusarium* sp. pose a serious threat to the Israeli avocado industry. *Phytoparasitica* **40**: 235-238.

Mucina L & Rutherford M (2006) *The vegetation of South Africa, Lesotho and Swaziland*. *Strelitzia* **19.** South African National Biodiversity Institute, Pretoria, South Africa.

Na F, Carrillo JD, Mayorquin JS, Ndinga-Muniania C, Stajich JE, Stouthamer R, Huang Y-T, Lin Y-T, Chen C-Y & Eskalen A (2018) Two novel fungal symbionts *Fusarium kuroshium* sp. nov. and *Graphium kuroshium* sp. nov. of Kuroshio shot hole borer (*Euwallacea* sp. nr. *fornicatus)* cause Fusarium dieback on woody host species in California. *Plant Disease* **102**: 1154-1164.

O'Donnell K, Sink S, Libeskind-Hadas R, Hulcr J, Kasson MT, Ploetz RC, Konkol JL, Ploetz JN, Carrillo D & Campbell A (2015) Discordant phylogenies suggest repeated host shifts in the *Fusarium–Euwallacea* ambrosia beetle mutualism. *Fungal Genetics and Biology* **82**: 277-290.

O'Donnell K, Libeskind-Hadas R, Hulcr J, Bateman C, Kasson MT, Ploetz RC, Konkol JL, Ploetz JN, Carrillo D & Campbell A (2016) Invasive Asian *Fusarium–Euwallacea* ambrosia beetle mutualists pose a serious threat to forests, urban landscapes and the avocado industry. *Phytoparasitica* **44**: 435-442.

Paap T, De Beer ZW, Migliorini D, Nel W & Wingfield MJ (2018) The polyphagous shot hole borer (PSHB) and its fungal symbiont *Fusarium euwallaceae*: a new invasion in South Africa. *Australasian Plant Pathology* **47**: 231-237.

Perrings C, Dehnen-Schmutz K, Touza J & Williamson M (2005) How to manage biological invasions under globalization. *Trends in Ecology & Evolution* **20**: 212-215.

Ploetz R, Pérez-Martínez J, Smith J, Hughes M, Dreaden T, Inch S & Fu Y (2012) Responses of avocado to laurel wilt, caused by *Raffaelea lauricola*. *Plant Pathology* **61**: 801-808.

Ploetz RC, Hulcr J, Wingfield MJ & De Beer ZW (2013) Destructive tree diseases associated with ambrosia and bark beetles: black swan events in tree pathology? *Plant Disease* **97**: 856-872.

Tyler G, Zheng Y, Kulinich M & Atamian H (2019) *Fusarium euwallaceae*: A Serious Threat to the Native and Ornamental Trees and Shrubs in Southern California. *Chapman University Student Scholar Symposium Abstracts And Posters.*

Umeda C, Eskalen A & Paine TD (2016) Polyphagous shot hole borer and Fusarium dieback in California. *Insects and Diseases of Mediterranean Forest Systems* **6**: 757-767. Springer.

van den Berg N, du Toit M, Morgan S, Fourie G & de Beer ZW (2019) First report of *Fusarium euwallaceae* causing necrotic lesions on *Persea americana*, in South Africa. *Plant Disease* **10** 10-18-1818-PDN.

Vettraino A, Roques A, Yart A, Fan J-t, Sun J-h & Vannini A (2015) Sentinel trees as a tool to forecast invasions of alien plant pathogens. *PLoS One* **10**: e0120571.

Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**: 294-299.

White EM, Wilson JC & Clarke AR (2006) Biotic indirect effects: a neglected concept in invasion biology. *Diversity and Distributions* **12**: 443-455.

Yin M, Duong TA, Wingfield MJ, Zhou X & De Beer ZW (2015) Taxonomy and phylogeny of the *Leptographium procerum* complex, including *Leptographium sinense* sp. nov. and *Leptographium longiconidiophorum* sp. nov. *Antonie van Leeuwenhoek* **107**: 547-563.

SUPPLEMENTARY INFORMATION

Table 1: The gene regions, primer sequences and names as well as the references that the primer pairs were sourced from, relevant to the study.

Gene Region	Primer names and sequences (5' to 3')	Reference
*TEF1*α	**EF1:** ATG GGT AAG GAG GAC AAG AC	(Karlsson *et al.*, 2016)
	EF2: GGA AGT ACC AGT GAT CAT GTT	
β-tubulin	**F-*βtub*1:** CAG GCT GGT CAA TGC GGT AAC CA	(Einax & Voigt, 2003)
	F-*βtub2*: GGA ATC CAT TCG ACG AA	
RBP2	**fRBP2 – 7cf:** ATG GGC AAG CAA GCC ATG GG	(Liu *et al.*, 1999)
	fRBP2 – 11ar: GCG TGG ATG TTG TCG TCG ACC	
COX1	**HCO 2198:** TAA ACT TCA GGG TGA CCA AAA AAT CA	(Vrijenhoek, 1994)
	LCO 1490: GGT CAA CAA ATC ATA AAG ATA TTG G	

Table 2: The measurements obtained from the lesion lengths, of the infected and control plants, the means per biological replicate and controls, as well as the overall means for the infected and control plants. These measurements were used to determine statistical significance of the results.

Plantlet number	Lesion lengths (cm)	Mean lesion lengths (cm)
1. Infected	3, 4, 3.6	3.53
2. Infected	3, 4, 2.9	3.33
3. Infected	6.3, 7, 4.5	5.93
4. Infected	3, 2.5, 6	3.83
5. Infected	6.5, 5.5, 4	5.33
6. Infected	7, 5, 4	5.33
7. Infected	3, 4, 7	4.67
		Overall mean (infected): 4.56
8. Control	2.1, 1.9, 1.9	1.97
9. Control	2.2, 1.8, 2	2
		Overall mean (controls): 1.99

Chapter 3: Evaluating avocado fruit cultivars for susceptibility to *Fusarium euwallaceae* and *Graphium euwallaceae* and testing potential chemical and biological control agents against *F. euwallaceae*

TABLE OF CONTENTS

ABSTRACT

The recent spread of the polyphagous shot hole borer (*Euwallacea fornicatus)*, ambrosia beetle, in South Africa, is of major concern to many facets of the country. This mycophagous, wood-boring beetle can maintain, transport and inoculate a variety of pathogenic symbionts, directly into host plant tissue, causing Fusarium dieback (FD). Since the initial report of this pest complex, on London Plane trees, in South Africa, sightings of this disease on other hosts have progressed significantly throughout the country and includes the report of this disease on *Persea americana* (avocado). Conventional agricultural practices are unequipped and broadly uninformed to effectively combat this pest and its associated disease and investigations towards trying to combat this disease are limited, since there is a lack of host-specific control means. Therefore, current studies are aimed at the selection of avocado cultivars that exhibit some level of resistance, together with the development of a chemical/biopesticide that be used to combat the disease, without compromising the viability and organic status of the plant's fruit yield. The objectives of this study were to assess the susceptibility of avocado fruit cultivars to the fungal symbionts associated with the PSHB and to further investigate chemical and biological control agents for their potential use to treat Fusarium dieback. This included the screening of two fungal associates of the PSHB, on different avocado cultivars. Additionally, the efficacy of different chemical and biological control agents, against *F. euwallaceae,* were assessed. Results found that an *in planta* co-inoculation with *Fusarium euwallaceae* and *Graphium euwallaceae* resulted in more severe symptoms than when inoculated on their own. Furthermore, all of the chemical agents that were tested in this study, indicated promising prospects, as their action led to a significant decrease in the growth of *F. euwallaceae* mycelia, *in vitro.* This study contributes

towards the management of Fusarium dieback, by indicating an increase in shared pathogenicity of the fungal associates of the PSHB and also addresses the high demand for potential chemical and biological agents for the treatment of Fusarium dieback, in South Africa.

INTRODUCTION

The routine spread of disease across the world, as a result of climate change and globalisation, means that many pathogenic organisms are introduced into areas where there is often little to no indication as to how these pathogens may adapt or react to their new environments (Perrings *et al.*, 2005). Historical and international perspectives on previous occurrences of such pathogens shed light on their potential impact. By considering the impact and resultant response by the environment and biotic communities, towards a newly established pathogen, it is often possible to better prime an area to the introduction of such pathogens and to predict the long-term implications (Ploetz *et al.*, 2013). As is the case with many newly introduced pathogens, vital cues towards the correct management and control of such pathogens can be gained from areas in which the disease has already established and its long-term implications have been researched and observed.

In the case of the beetle-fungus complex between *E. fornicatus* and its associated symbionts, its presence in the United States of America, Israel and recently in South Africa, has indicated the potential hazard to the global agricultural framework, particularly to avocado growing industries (Eskalen *et al.*, 2013, Freeman *et al.*, 2013, van den Berg *et al.*, 2019). In these countries, where this beetle and other ambrosia beetles, are considered highly-invasive threats, research towards limiting the associated disease risks has indicated many difficulties and complexities. The

research generated by the international scientific community towards all ambrosia beetles and their associated diseases is invaluable and allows for the development and modification of existing control strategies, for local communities.

The response and means for control for most ambrosia beetles follow some universal, fundamental pathways to attempt to limit their potential damage. It is important to note that all available control means must be considered, even if previous research has indicated that specific approaches are situationally ineffective. This is because pathogens may respond differently, in unlike areas and certain control measures are more/less suitable or viable based on the country and the way the agricultural structure is managed. Differences can include diverse regulations or standards regarding the use of chemical and biological control agents (Sundh & Goettel, 2013), differences in density and value of crop plantations (Evans *et al.*, 2010), availability of funding towards persistent studying of a disease as well as the skills and expertise available to manage and/or study the disease.

Ambrosia beetles and associated diseases are often considered to be anomalous and exceptionally difficult to manage and control (Castrillo *et al.*, 2013). However, scrutinising the complexity of the disease lifestyle means that there are more potential aspects that can be targeted for disruption or interference. In the case of a vector-borne disease, such as Fusarium dieback (FD), prospects for the application of control means could encompass a combination of alterations and adaptations to conventional farming practices, the selection of- or breeding for less susceptible plant material (Niks *et al.*, 2011), the control of the insect vector itself and/or the control of one or more of the associated symbionts (Jones *et al.*, 2017).

To date, there has been no selection or breeding for crop varieties that have resistance to FD. For avocado, only a single study has been performed to investigate differences in susceptibility, among avocado cultivars, to the PSHB (Jones & Paine, 2017). The results of which indicated different attack rates and rates of gallery formation among the various cultivars, indicating potential differences in susceptibility to PHSB of the commercial cultivars.

Nonetheless, currently-existing agricultural practices are aimed at limiting the spread of PSHB and rely on the use of timely and effective removal of sources/reservoirs in the form of infested woody plant material (Hulcr & Stelinski, 2017). The insect-vector may be further controlled through the application of chemical pesticides (Jones & Paine, 2018). These approaches are generally not aimed at treating or diagnosing already infested trees, but rather towards limiting future spread and to reduce chances for infection of uninfected plants. Insecticides are generally ineffective at controlling ambrosia beetles inside already infested trees, as their action can be nullified by the lifestyle of these beetles since they spend almost their entire lives within their host tree (Atkinson *et al.*, 1988). Most ambrosia beetles are also exclusively mycophagous, not phloeophagous, meaning systemic insecticides are also generally ineffective, at any given time during any life stage (Atkinson *et al.*, 1988).

While it is important to re-consider new control strategies for the PSHB insect-vector, to limit autonomous spreading, it is equally important to consider the use of agents to inhibit or prevent the growth of the PSHB fungal symbionts, responsible for the causation of FD (Jones *et al.*, 2017), especially for trees that have already been infected. Initial studies have shown promising *in planta* results for the application of broad-spectrum surfactant trunk sprays and injections such as triazole fungicides

(Jones *et al.*, 2017, Mayorquin *et al.*, 2018). Biological control, in the form of culture powder applications, has also been investigated for potential use against the fungal associates of the PSHB (Muis, 2016). Members of the *Bacillus* and *Trichoderma* genera have been extensively considered for their use as biological control agents and a recent study found avocado rhizobacteria with antifungal activity against the pathogens responsible for FD (Guevara-Avendaño *et al.*, 2018).

This study aimed to examine and evaluate the pathogenicity of the causal agent for FD – *F. euwallaceae,* specifically the isolate that was isolated and identified from avocado in Sandton, Gauteng, in South Africa, in **Chapter 2**, (*F. euwallaceae,* isolate CMW 51808) on available avocado material. This included the use of clonal avocado plantlets, from two previously untested avocado cultivars. In addition, this study also set out to determine whether one of the other persistently, associated fungal symbionts (*G. euwallaceae*) was able to cause disease on avocado, on its own and if so, to what degree. These two fungal organisms were then also used in a co-inoculation study to determine whether the combination inoculation resulted in disease symptoms that were equal to or more severe than the sum of the effects caused by the individual organisms.

Together with the pathogenicity trials, the efficacy of several chemical control agents, in the form of broad-spectrum fungicides were applied, at different concentrations, *in vitro,* to determine the possibility of their use, to achieve complete and practical mycelial inhibition of *F. euwallaceae.* Furthermore, dual-culture assays with *F. euwallaceae* and some commonly used biological control agents, namely *Bacillus* spp. and *Trichoderma* spp., were conducted to observe the nature of their interactions and

the possible feasibility of their applications as control measures, to limit the growth and subsequent damage caused by *F. euwallaceae.*

MATERIALS AND METHODS

Preparation of inoculum for pathogenicity trials

½ PDA nutrient medium was prepared as described in **Chapter 2**. A heat-sterilised needle tip was used to transfer the mycelia of already cultured *F. euwallaceae* (isolate CMW 51808) to the surface of the nutrient media. A confirmed isolate of *G. euwallaceae,* obtained from FABI (Forestry and Agriculture Biotechnology Institute), isolated from *Acer negundo* (Box Elder), was also plated out in replicates, in the same way. These culture plates were incubated in the dark, at 25°C, for 5 days and used in downstream pathogenicity trials and control assays.

Pathogenicity trial using PSHB associates on clonal avocado plantlets

Live, clonal avocado plantlets were obtained for two available and previously untested avocado cultivars, namely Edranol and Ettinger. A total of 22 plantlets, for each cultivar, were used. However, the plantlets for each cultivar differed substantially from the plantlets of the other cultivar. The seedlings for the Edranol cultivar were about 8 months old and about 60 cm in height, whereas the Ettinger seedlings were about 18 months old and about 1.5 m in height. Therefore no accurate comparisons, between differences in susceptibility, could be made between the cultivars.

Shallow excisions were made on the stems of the plantlets, using a cork borer. The freshly wounded tissue was then inoculated with agar plugs (3 mm in diameter) colonised by freshly grown *F. euwallaceae* only, *G. euwallaceae* only and a co-

inoculation of both fungi. Each trial included three control plants – inoculated with sterile agar plugs, five plants co-inoculated with both *F. euwallaceae* and *G. euwallaceae,* seven plants inoculated with *F. euwallaceae* alone and seven plants inoculated with *G. euwallaceae* alone. The wounds were wrapped with parafilm and the plants were left to incubate in a greenhouse, for 6 weeks at 25°C. The plants were watered twice per week and fertilised once a week with Wonder Nitrosol® (Efekto).

After 6 weeks, the plants were photographed, the parafilm was removed and lesions were observed and their lengths were measured. In addition to measuring the size of the lesions, primary isolations were made from the edges of the lesions (infected and control plants), to recover *F. euwallaceae* and *G. euwallaceae* respectively, from the infected plants, as well as to ensure their absence from the control plants. The resultant isolates were used as the basis for DNA extraction and subsequent PCR amplification of the translation elongation factor 1α (*TEF1*α) using the EF1/EF2 primer set. The thermal conditions for the PCR were 5 min at 95°C for initial denaturation, 40 cycles of 1 min at 94°C, 1 min at 67°C, and 1 min at 72°C for annealing and then a final 10 min at 72°C for the final extension stage. The holding temperature was set to 4°C. The products were submitted for sequencing at DNA Sanger sequencing facility in the Faculty of Natural and Agricultural Sciences, University of Pretoria (UID:78566) and analysis of the sequence data re-confirmed the identity of the recovered fungal organisms from the inoculated plants.

In vitro control assay on Fusarium euwallaceae, using broad-spectrum fungicides

Based on literature and findings by Mayorquin *et al.* (2018), four fungicides were tested for their ability to inhibit the growth of *F. euwallaceae* mycelia. Three triazole fungicides

namely metconazole (Sigma-Aldrich), tebuconazole (Sigma-Aldrich) and propiconazole (Sigma-Aldrich), along with another broad-spectrum fungicide fluazinam (Cheminova) were chosen for this study. The use of all the fungicides were based on the *in planta* EC50 (Effective Concentration of a chemical that gives half-maximal response) described by Mayorquin *et al.* (2018) and were used as the baseline concentrations, from which two higher concentrations followed, towards trying to achieve complete mycelial inhibition. The three concentrations for each chemical agent are shown in **Table 1**, in the supplementary information. To achieve the desired concentrations, the fungicides were weighed out in a 15 ml conical tube, using a precision analytic balance. Once the desired weight to volume was achieved, cooled ½ PDA nutrient medium was amended with the heat-sensitive fungicides. The contents were mixed using a vortex and then added to the remaining media. The amended media was poured into 90 mm Petri-plates, each marked perpendicularly, with lines, crossing through the centre of the base of the Petri-plates.

Thereafter, an agar plug, colonised by *F. euwallaceae,* was placed upside down on the surface of amended media, in the centre of each of the perpendicular intersections. Control plates were also prepared by placing an agar plug, colonised by *F. euwallaceae,* onto the surface of unamended ½ PDA medium. The plates were wrapped with parafilm and incubated in the dark, at 25°C, for 3 days. After incubation, the margins of the mycelial growth were marked on the lines, previously added to the base of the Petri-plates. The distance between each mark, on each line, was measured and an average mycelial growth diameter was recorded for 3 DAP (Days After Plating). The Petri-plates were then again incubated in the dark, at 25°C, for a further 3 days. Markings were made and average mycelial growth diameter was recorded for 6 DAP.

***In vitro control assay on Fusarium euwallaceae, using Bacillus* spp.**

Petri-plates containing unamended ½ PDA were each marked perpendicularly, with lines, crossing through the centre of the base of the Petri-plates. ½ PDA plugs, colonised by *F. euwallaceae,* were placed in the centre of each of the perpendicular intersections. Together with placing the *F. euwallaceae* colonised plugs, one granule of *Bacillus* spp. culture powder was added to the four polar directions on the nutrient medium Petri-plates. These biological control agents are available as commercial formulations and are intended for direct application as a powder culture. Two *Bacillus* culture powders were obtained from the DSC Laboratory, in South Africa: B-Rus (containing *Bacillus sublitis* and *Bacillus amyloliquefaciens* subsp. *plantarum* CH13) and Mity-Gro (containing *Bacillus laterosporus*). Control plates were also prepared by placing an agar plug, colonised by *F. euwallaceae* onto the surface of sterile ½ PDA medium, without any *Bacillus* spp. culture powder. Control plates for the *Bacillus* culture powder were also prepared by placing a granule of the culture powder onto the surface of ½ PDA medium. The Petri-plates were wrapped with parafilm and incubated in the dark, at 25°C, for 3 days. After incubation, the average mycelial growth diameter was recorded for 3 DAP and for 6 DAP.

***In vitro control assay on Fusarium euwallaceae, using Trichoderma* spp.**

Petri-plates containing unamended ½ PDA were each marked 2 cm from the sides of the base of the Petri-plates and a line was drawn through the middle of the plate, connecting the two markings. To the one side of the line drawn, a *F. euwallaceae* colonised agar plug was placed, upside down, onto the surface of the medium and to the other side of the line, a *Trichoderma* spp. colonised agar plug was added. Control plates for the *Trichoderma* spp. culture were also prepared by placing a *Trichoderma*

spp. colonised agar plug onto the surface of sterile ½ PDA medium. Control plates were also prepared for *F. euwallaceae* by placing a colonised agar plug onto the surface of ½ PDA medium. The Petri-plates were wrapped and incubated in the dark, at 25°C, for 3 days. After incubation, the margins of the *F. euwallaceae* mycelial growth were marked and the mycelial growth diameter was recorded for 3 DAP and for 6 DAP.

Statistical analysis of pathogenicity trial and control assay data

The data from the pathogenicity trial and *in vitro* assays were analysed, using the GraphPad Prism 8 software package. The statistical analysis was carried out similarly for all the data and was aimed at determining standard error and significance of the data with a 95% confidence interval ($\alpha = 0.05$), compared to the data generated by the control groups of each experiment. The overall arithmetic means ($\bar{x}$), standard deviations as well as the standard errors for each data set were calculated. The statistical analysis of the data was analysed by firstly determining whether the data set showed Gaussian (normal) distribution, by evaluating the normality and lognormality of the data set, using four tests: The Anderson-Darling test, The D'Agostino & Pearson test, The Shapiro Wilk test and the Kolmogorov-Smirnov test. When assuming Gaussian distribution, according to the Shapiro Wilk test, an ordinary, one-way-ANOVA with Tukey's multiple comparisons test was done to determine the significance of the data compared to that of the control group. In the case that the data was not normally distributed, a non-parametric test was done, in the form of the Kraskal-Willis test with Dunn's multiple comparisons test, to determine the significance of the data compared to that of the control group. Bar graphs, with bars indicative of overall arithmetic means ($\bar{x}$), were constructed according to the data and statistical analyses generated.

RESULTS

Pathogenicity trial using PSHB associates on clonal avocado plantlets

Following the incubation of the fungal-inoculated clonal avocado plantlets, water-soaked lesions were observed on both cultivars, as expected, after 6 weeks. The control plants however retained a healthy appearance and upon removing the parafilm, dry, brown lesions were observed at the points of inoculation, consistent with a response to wounding. The fungal-inoculated plants showed external symptoms of disease including wilting and slight discoloration and drooping of the foliage. Internal symptoms of the infected plants included clear reddish-brown pigmentation, in the vascular tissue and water-soaked lesions. No sugary exudates were observed. The results of this trial proved that the average lesion length recorded for the *F. euwallaceae* was significantly larger than that recorded for the average lesion length recorded for the *G. euwallaceae,* in both cultivars. Further, the co-inoculation of both fungi yielded lesions significantly larger than the sum of both the lesions caused by *F. euwallaceae* alone and *G. euwallaceae* alone, for both cultivars.

The statistical analysis of the average lesion length compared to the average lesion length of the controls indicated that *F. euwallaceae* alone, *G. euwallaceae* alone and co-inoculation of both fungi yielded lesions that were significantly different from the controls. The statistical analysis also revealed that there were significant differences between the average lesion measurements from the fungal-inoculated plantlets. This means that *F. euwallaceae* formed lesions larger than the lesions caused by *G. euwallaceae,* implying that *F. euwallaceae* is more pathogenic. Nonetheless, the significant difference between the *G. euwallaceae* inoculated plantlets and the controls suggests that *G. euwallaceae* is pathogenic when inoculated on its own and further

suggests synergistic pathogenicity with *F. euwallaceae*, to cause further tissue damage and colonisation of host tissue.

Primary isolations were performed, onto ½ PDA nutrient medium, but neither *F. euwallaceae* nor *G. euwallaceae* were re-isolated from the control plants. In contrast, the pathogen inoculated and co-inoculated plants appeared less healthy than when they were first inoculated and primary isolations from all symptomatic sites re-isolated *F. euwallaceae* and *G. euwallaceae* respectively. Images from the pathogenicity trial are presented in **Figure 1** and **Figure 2.** The average lesion lengths and the statistical significance thereof are illustrated in **Figures 3 and 4.** The quantitative data from the trial and statistical analysis thereof is indicated in **Table 2 – 5**, in the supplementary information**.**

	Nature of inoculation on Ettinger plantlets			
	Fusarium euwallaceae alone	*Graphium euwallaceae* alone	Co-inoculation of both fungi	Controls
Wound/Lesion				
Area Under Wound/Lesion				
Overall Plant Health				

Figure 1: The plantlets for the Ettinger avocado cultivar, from the pathogenicity trial, inoculated with the fungal associates of the PSHB indicating disease symptoms of Fusarium dieback, absent in the control plants. The plantlets, inoculated with *Fusarium euwallaceae* only, showed drooped leaves presenting a dull green colour – accounted for a decrease in the efficiency of the water-conducting vascular tissue, from successful infection of *F. euwallaceae.* Upon removal of the parafilm, brown, water-soaked lesions were observed on the pathogen-inoculated stems. Internal reddish-brown lesions visibly extended into the vascular tissue. Similar results can be observed for the *Graphium euwallaceae* only inoculated plantlets, indicating towards the standalone pathogenicity of *G. euwallaceae*. The average lesion length for the plants inoculated with *G. euwallaceae* was significantly less than what was observed for the *F. euwallaceae* only inoculation plantlets but significantly more than the control plants. The plantlets co-inoculated with both fungi were visibly the most affected out of all the plants from the entire trial. Most of the leaves of the co-inoculated plants were drooped but remained attached, presenting a dull green-yellow colour. Internal reddish-brown lesions extended considerably more than the lesions for the other groups. The control plantlets responded to the artificial wounding by producing a dried-out lesion and stripping away of the plant tissue around the wounds showed a clear absence of pathogenic infection. The leaves on the control plants were noticeably healthier than the leaves for the pathogen-inoculated plants.

	Nature of inoculation on Edranol plantlets			
	Fusarium euwallaceae alone	*Graphium euwallaceae* alone	Co-inoculation of both fungi	Controls
Wound/Lesion				
Area Under Wound/Lesion				
Overall Plant Health				

Figure 2. The lesions on the fungal-inoculated plantlets, for the Edranol cultivar, showing disease symptoms that were not observed in the control plants. Upon removal of the parafilm, dry, brown lesions, were observed for the control plants as a response to the artificial wounding, whereas water-soaked, lesions were observed for all the inoculated plantlets and further internal reddish-brown discolouration was observed extending into the vascular tissue, comparable to the results obtained from for the other cultivar. *Graphium euwallaceae* caused lesions that were significantly smaller than what was observed for the *F. euwallaceae* only inoculation, but with sufficient pathogenicity to cause disease symptoms, when inoculated on its own. The plantlets co-inoculated with both fungi revealed lesions that were significantly larger than the lesions formed by the standalone inoculations, of the two fungi.

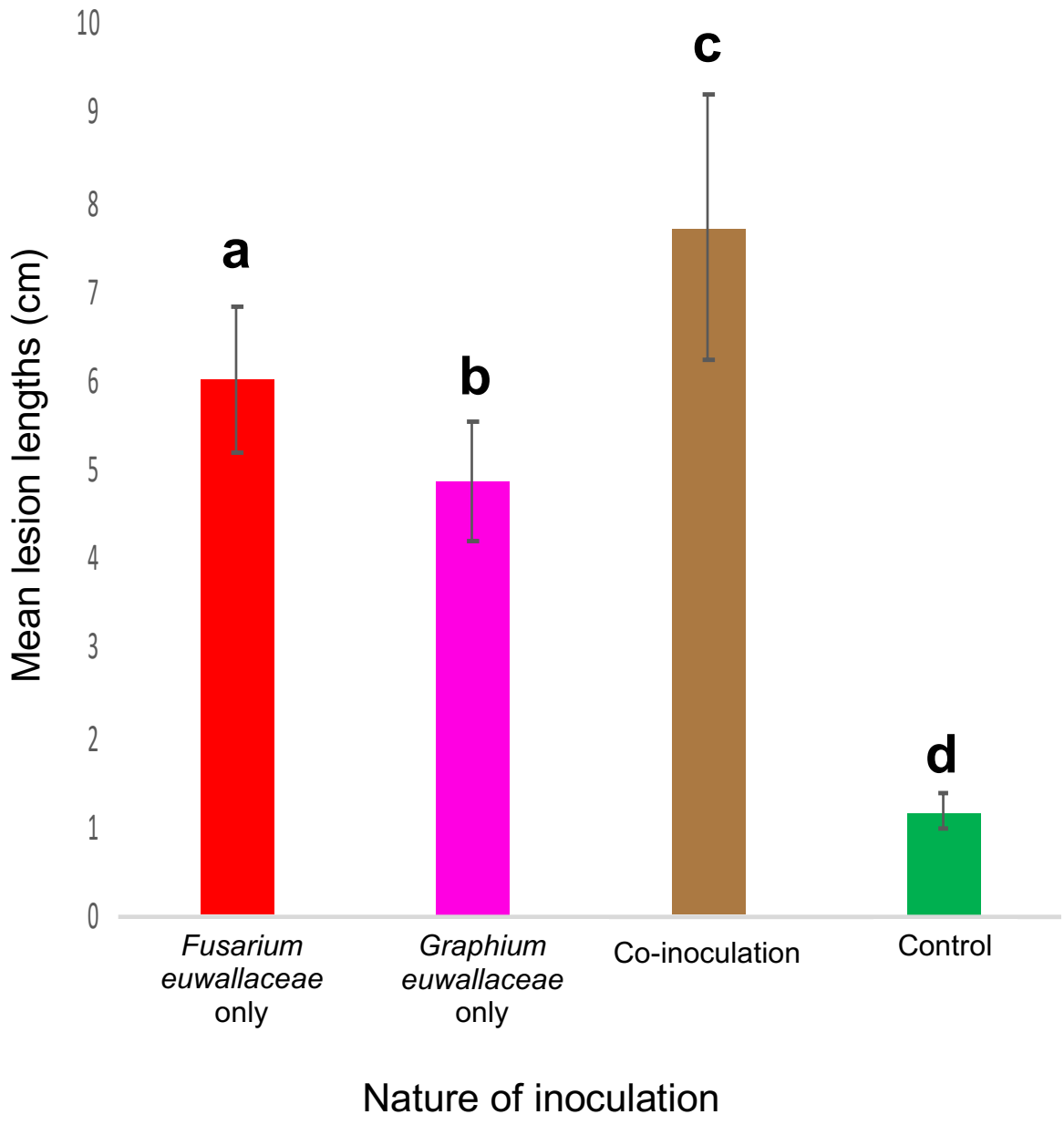

10
9
8
7
6
5
4
3
2
1
0
Mean lesion lengths (cm)
a
b
c
d
Fusarium euwallaceae only
Graphium euwallaceae only
Co-inoculation
Control
Nature of inoculation

Figure 3: The mean measurements of the lesions obtained from the Ettinger plantlets, after being inoculated with the fungal associates of the PSHB, as well as the control plants, at 6 weeks after incubation. The mean measurements include the lesions from *Fusarium euwallaceae* only inoculations ($\bar{x}$ = 6 cm, in red), the lesions from *Graphium euwallaceae* only inoculations ($\bar{x}$ = 4. 87 cm, in pink), the co-inoculation of both fungi ($\bar{x}$ = 7.72 cm, in brown) and the control plantlets ($\bar{x}$ = 1.17 cm, in green). Each bar indicates the mean and standard error of the lesion measurements. The data followed Gaussian distribution and an ordinary ANOVA with Tukey's multiple comparisons test revealed that there was a significant difference between the lesions from the different groups, indicated by the different letters. The mean lesion lengths of the fungal-inoculated plantlets (a, b) including the co-inoculation (c), differed significantly from the mean lesion lengths of the control plants (d). Statistical analysis also revealed significant differences in the average lesion lengths between the three inoculation groups, including significant differences between the single pathogen inoculations (a and b) as well as significant differences between the single pathogen inoculations (a, b) and the co-inoculations (c). Bars represented with the same letter are not significantly different at $P < 0.05$.

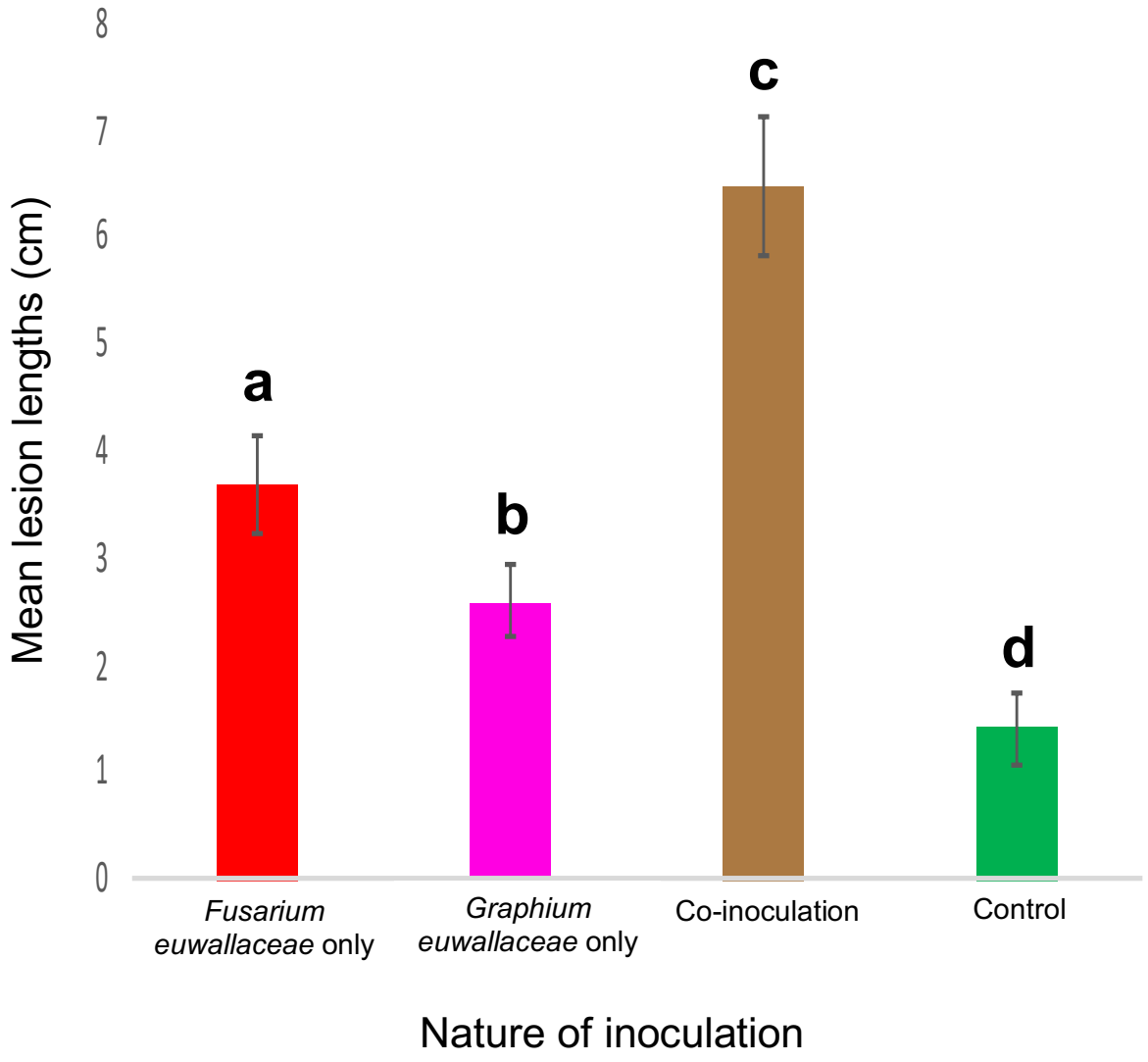

Mean lesion lengths (cm)
8
7
6
5
4
3
2
1
0
a
b
c
d
Fusarium euwallaceae only
Graphium euwallaceae only
Co-inoculation
Control
Nature of inoculation

Figure 4: The mean measurements, recorded at 6 weeks after incubation, of the lesions obtained from the Edranol plantlets after being inoculated with the fungal associates of the PSHB, as well as the control plants. The mean measurements include the lesions from *Fusarium euwallaceae* only inoculations ($\bar{x}$ = 3.69 cm, in red), the lesions from *Graphium euwallaceae* only inoculations ($\bar{x}$ = 2.69 cm, in pink), the co-inoculation of both fungi ($\bar{x}$ = 6.46 cm, in brown) and the control plantlets ($\bar{x}$ = 1.40 cm, in green). The bars indicate the mean and standard error of the lesion measurements. The data followed normal distribution and multiple comparisons tests revealed significant differences between the fungal-inoculated (a, b, c) and control plants (d). Further analysis also revealed significant differences between the three inoculation groups including significant differences between the single pathogen inoculations (a, b) and the co-inoculations (c). The lesions formed, on the co-inoculated plantlets for this trial, proved to be drastically different from the control plants and the single pathogen inoculations. Bars represented with the same letter are not significantly different at $P < 0.05$.

In vitro control assay on Fusarium euwallaceae, using broad-spectrum fungicides

- ***Testing the effectivity of fluazinam against Fusarium euwallaceae mycelia***

The results for the fluazinam assay indicated that the minimum concentration (0.05 µg/ml) slowed the growth of the *F. euwallaceae* mycelia significantly when compared to the control plates, at 3 DAP. As expected, the two higher concentrations that followed, proved to further decrease the growth speed of the mycelia, with the slowest mycelial growth observed at the highest concentration (0.15 µg/ml). This assay also proved that fluazinam lost some effectivity throughout this experiment, as *F. euwallaceae* mycelia continued to grow on plates containing all three concentrations. However, fluazinam remained partially effective throughout this experiment, as the mean growth of the *F. euwallaceae* mycelia, from all the amended plates, differed significantly from the controls, even at 6 DAP. Images for these results are shown in **Figure 5** and the mean mycelial growth, standard error and significance thereof are illustrated in **Figure 6** and **Figure 7.** Complete mycelial inhibition was not observed for the three concentrations used for fluazinam but it is likely that slightly increasing the highest concentration tested, from 0.15 µg/ml to 0.2 µg/ml, would result in complete inhibition, *in vitro*. The quantitative data and statistical analysis of the results from this assay are shown in **Tables 6 – 9**, in the supplementary information.

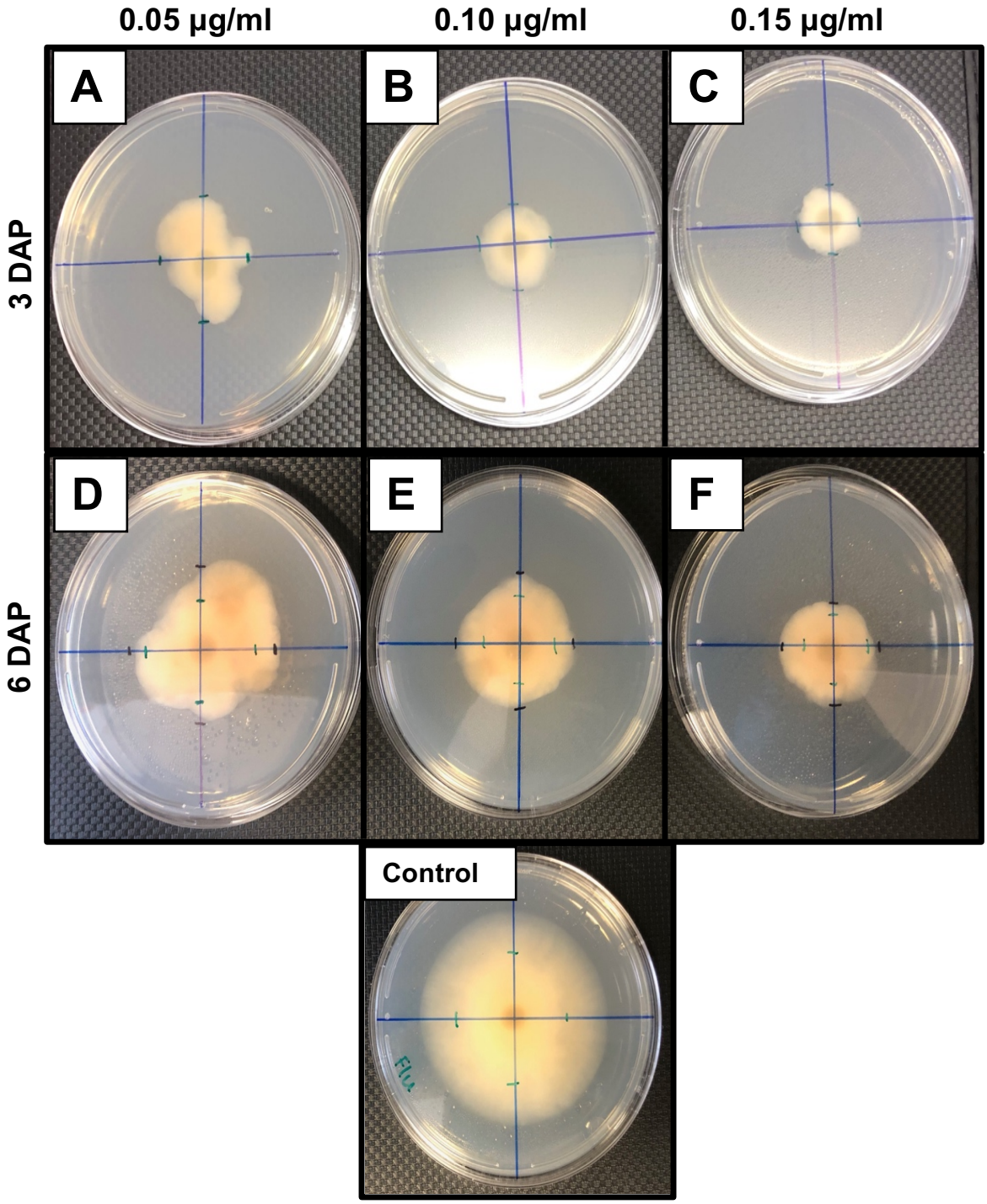


Figure 5: The culture morphology of *Fusarium euwallaceae*, plated on ½ PDA nutrient medium, amended with fluazinam. The images labelled A – C indicate the plates at 3 DAP (days after plating) and the images labelled D – F indicate the plates at 6 DAP. A and D indicate the plates at 0.05 µg/ml, B and E indicate the plates at 0.10 µg/ml, while C and F indicate the plates at 0.15 µg/ml, amended with fluazinam. The appearance of a control plate – *F. euwallaceae* plated on sterile, unamended, ½ PDA medium, at 6 DAP, is also shown here.

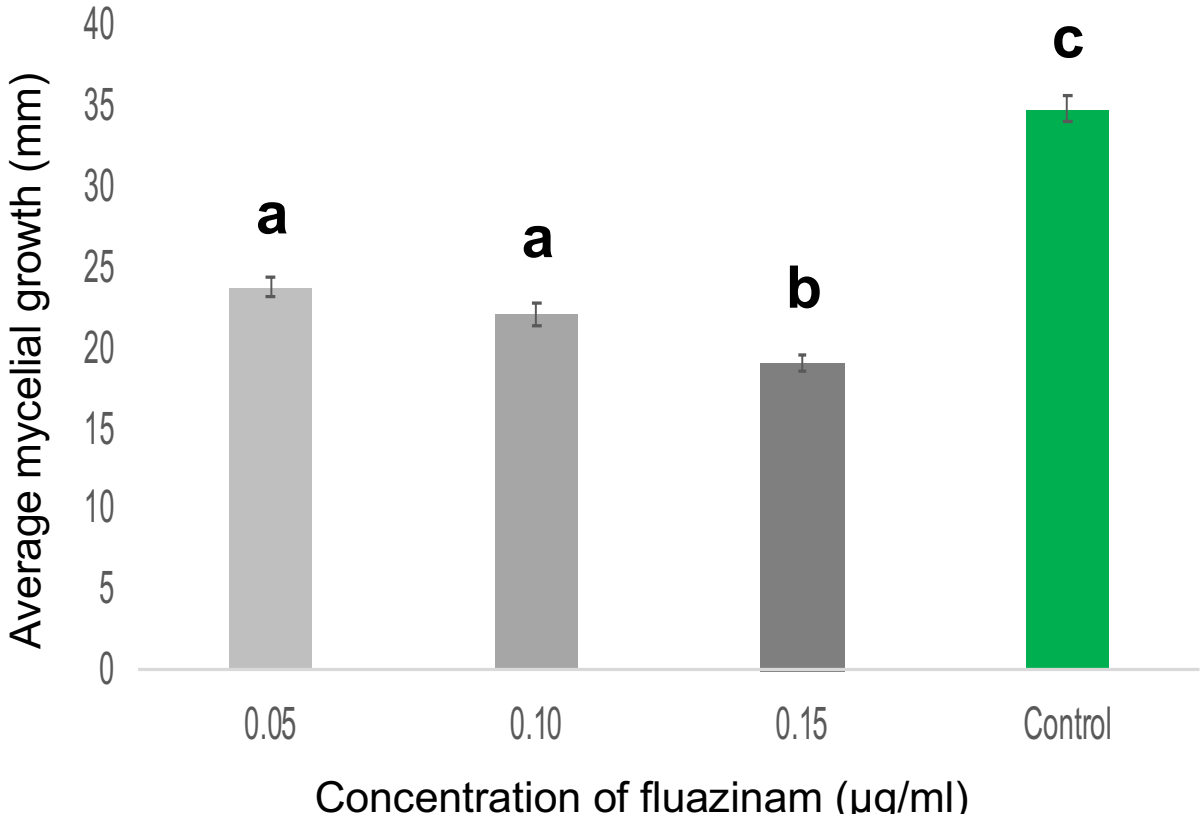


Figure 6: The mean measurements of *Fusarium euwallaceae* mycelia, plated onto different concentrations of ½ PDA, amended with fluazinam, at 3 DAP. The bars indicate the mean and standard error of the mycelial growth of the eight replicates. The data was normally distributed and multiple comparisons tests revealed significant differences between the *F. euwallaceae* average mycelial growth on the various concentrations of the chemically amended plates (a, b) as well as significant differences between the unamended control plates (c) and the amended plates (a, b). These significant differences are indicated with different letters at $P < 0.05$. The significance in difference, between the lowest concentration and the highest concentration, indicates an increase in effectivity as the concentration is increased and the difference between and the amended plates (a, b) and the controls (c) means that fluazinam was effective at inhibiting the growth of *F. euwallaceae* mycelia, even at the lowest concentration.

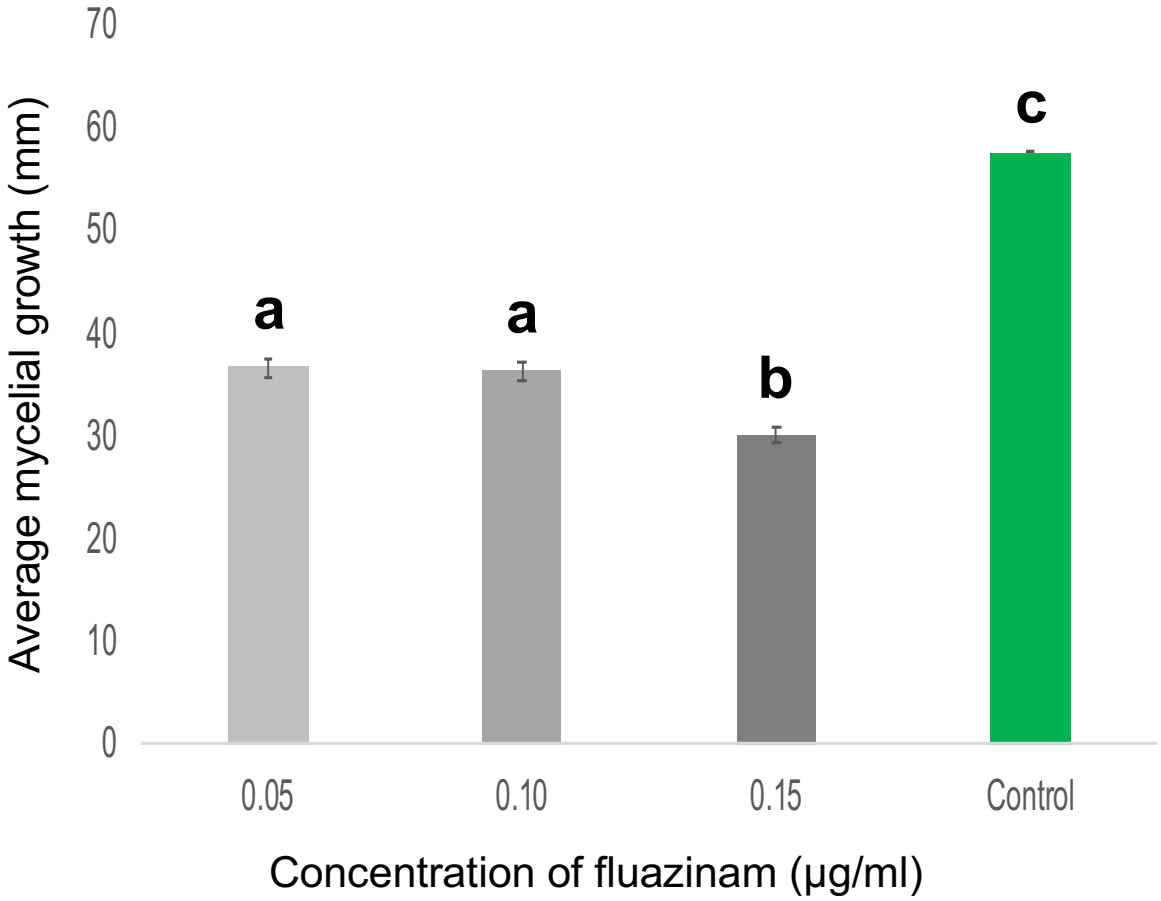


Figure 7: The mean measurements of *Fusarium euwallaceae* mycelia, plated onto nutrient media amended with fluazinam, at 6 DAP. Each bar indicates the mean and standard error of the mycelial growth of the eight replicates, The data was normally distributed and a multiple comparisons tests showed significant differences between the *F. euwallaceae* average mycelial growth on the various concentrations of amended plates (a and b). The analysis also revealed significant differences between the unamended control plates (c) (*F. euwallaceae* plated on unamended, ½ PDA medium) and the amended plates (a, b). Bars represented with the same letter are not significantly different at $P < 0.05$. At 6 DAP, the average mycelial growth, from the amended plates, still differed significantly from the control plates, indicating sustained partial effectivity. No complete mycelial inhibition was observed at any of the

concentrations, rather, it appeared as if the efficacy of fluazinam decreased slightly as time progressed.

- ***Testing the effectivity of metconazole against Fusarium euwallaceae mycelia***

Complete mycelial inhibition was observed for the three concentrations used for metconazole (25 µg/ml (*in planta* EC50), 40 µg/ml and 60 µg/m). The assay also proved that metconazole remained effective throughout this experiment, as complete mycelial inhibition was observed, even at 6 DAP. Images for these results are shown in **Figure 8**. Since complete mycelial inhibition was observed at the lowest applied concentration, no mycelial growth measurements could be recorded.

- ***Testing the effectivity of tebuconazole against Fusarium euwallaceae mycelia***

Complete inhibition of the *F. euwallaceae* mycelia was observed for the three concentrations used for tebuconazole (100 µg/ml (*in planta* EC50), 150 µg/ml and 200 µg/m). The assay also proved that tebuconazole, similarly to metconazole, remained effective throughout this experiment, as complete mycelial inhibition was observed, even at 6 DAP. Images for these results are shown in **Figure 9**. Complete mycelial inhibition meant that no mycelial growth measurements could be recorded.

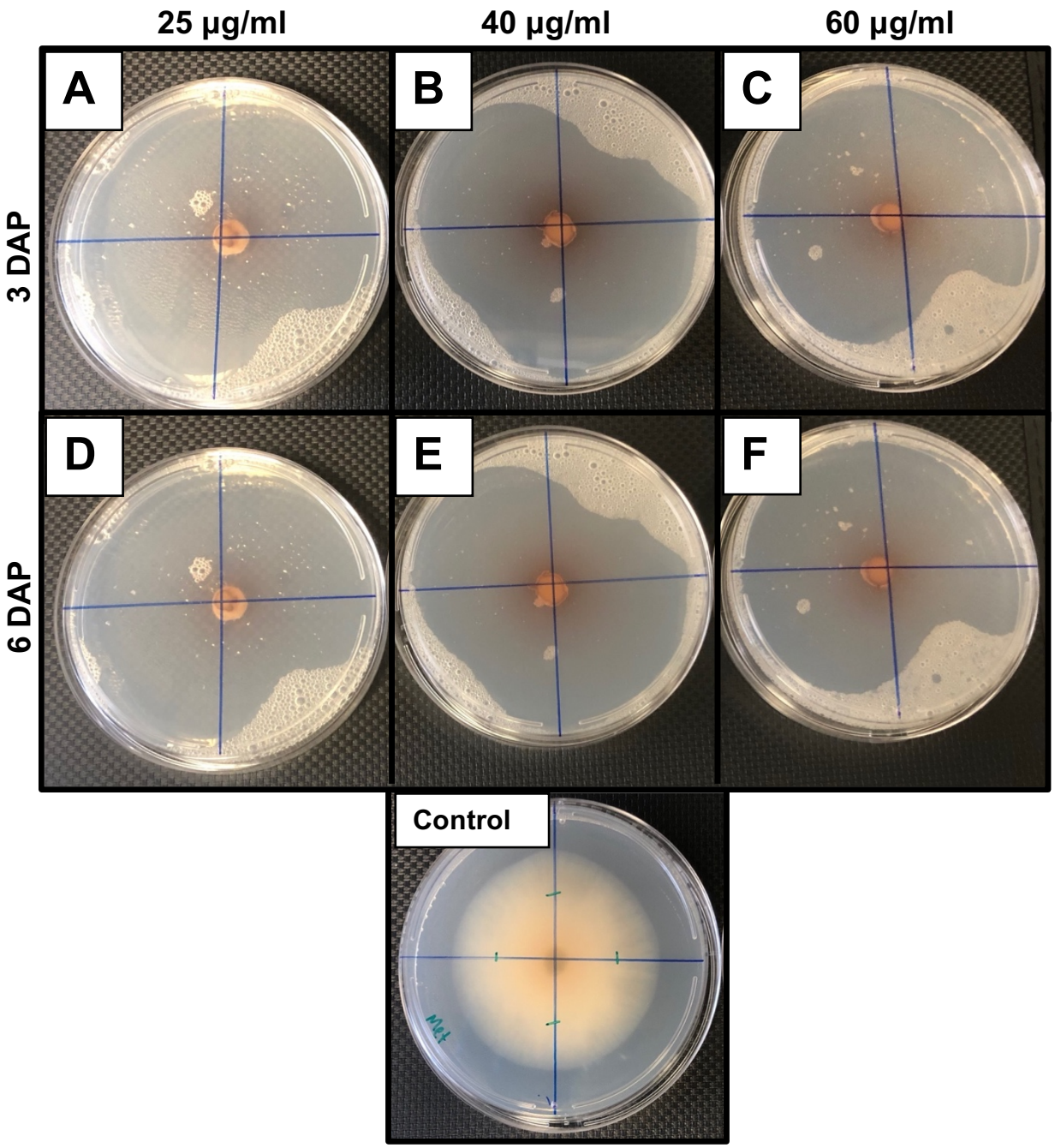


Figure 8: The culture morphology of *Fusarium euwallaceae*, plated on ½ PDA nutrient medium, amended with metconazole. The images labelled A – C indicate the plates at 3 DAP (days after plating) and the images labelled D – F indicate the plates at 6 DAP. A and D indicate the plates at 25 µg/ml, B and E indicate the plates at 40 µg/ml, while C and F indicate the plates at 60 µg/ml, amended with metconazole. The appearance of a control plate – *F. euwallaceae* plated on sterile, unamended, ½ PDA medium, at 6 DAP, is also shown here. Complete mycelial inhibition can be observed on all the plates, except for the control plate.

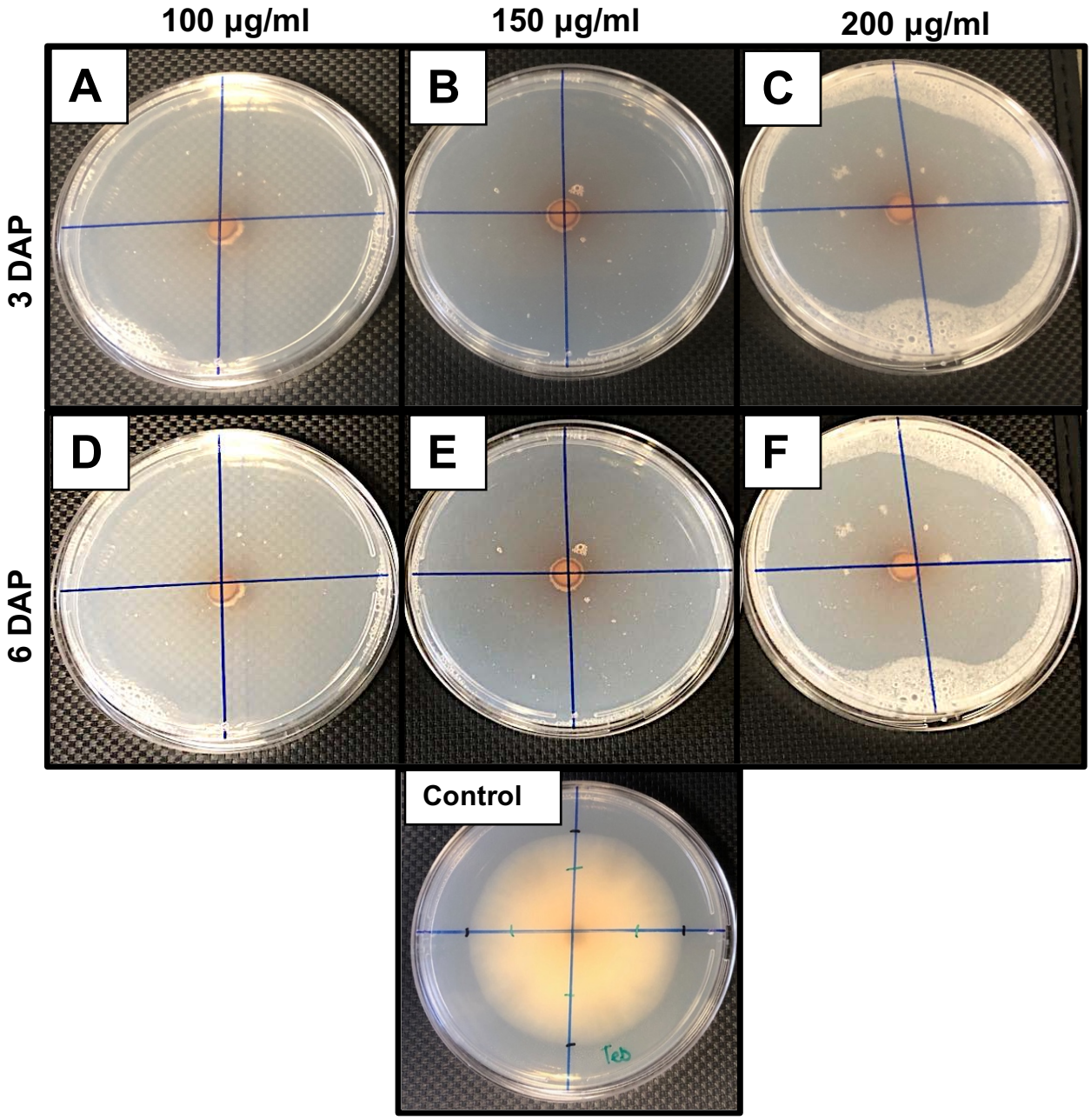


Figure 9: The morphology of *Fusarium euwallaceae*, plated on nutrient medium, amended with tebuconazole. The images labelled A – C indicate the plates at 3 DAP (days after plating) and the images labelled D – F indicate the plates at 6 DAP. A and D indicate the plates at 100 µg/ml, B and E indicate the plates at 150 µg/ml, while C and F indicate the plates at 200 µg/ml, amended with tebuconazole. The appearance of a control plate – *F. euwallaceae* plated on sterile, unamended, ½ PDA medium, at 6 DAP, is also shown here. Complete mycelial inhibition was observed for all the chemically amended plates, even at 6 DAP.

- ***Testing the effectivity of propiconazole against* Fusarium euwallaceae *mycelia***

In contrast to the complete mycelial inhibition that was observed for the other two triazole fungicides, at all three concentrations, the results for the propiconazole assay indicated that the minimum concentration (150 µg/ml) showed actively growing *F. euwallaceae* mycelia but differed significantly, compared to the average mycelial growth of to control plates, at 3 DAP. As expected, the two higher concentrations (350 µg/ml and 500 µg/ml) that followed, proved to further decrease the growth of the mycelia, with complete mycelial inhibition being observed at 500 µg/ml, at 3 DAP. Similarly to fluazinam, this assay also proved that propiconazole lost some effectivity throughout this experiment, as actively growing *F. euwallaceae* mycelia continued to grow at the two lower concentrations and started to appear, on the 500 µg/ml concentration plates, at 6 DAP. Complete mycelial inhibition was therefore only achieved for the highest concentration that was applied and was proven to lose effectivity soon thereafter. However, propiconazole remained partially effective throughout this experiment, as the mean growth of the *F. euwallaceae* mycelia differed significantly from the controls, even at 6 DAP. Images for these results are shown in **Figure 10** and the mean mycelial growth, standard error and significance thereof are illustrated in **Figure 11** and **Figure 12.** The quantitative data and statistical analysis of the results from this assay are shown in **Tables 10 – 13,** in the supplementary information.

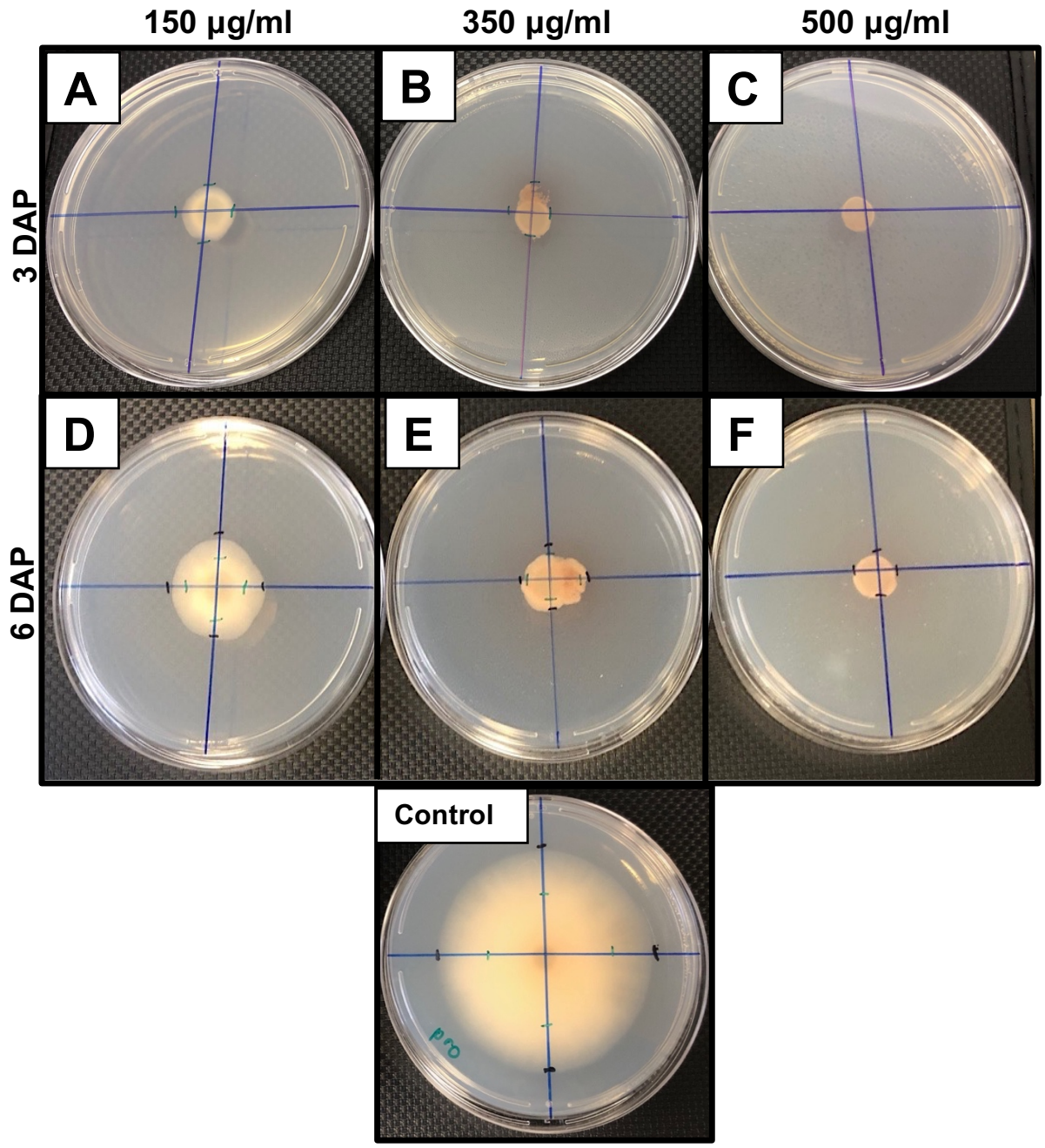


Figure 10: The culture morphology of *Fusarium euwallaceae*, plated on ½ PDA nutrient medium, amended with propiconazole. The images labelled A – C indicate the plates at 3 DAP (days after plating) and the images labelled D – F indicate the plates at 6 DAP. A and D indicate the plates at 150 µg/ml, B and E indicate the plates at 350 µg/ml, while C and F indicate the plates at 500 µg/ml, amended with propiconazole. Complete mycelial inhibition can be observed on plate C. The appearance of a control plate – *F. euwallaceae* plated on sterile, unamended, ½ PDA medium, at 6 DAP, is also shown here.

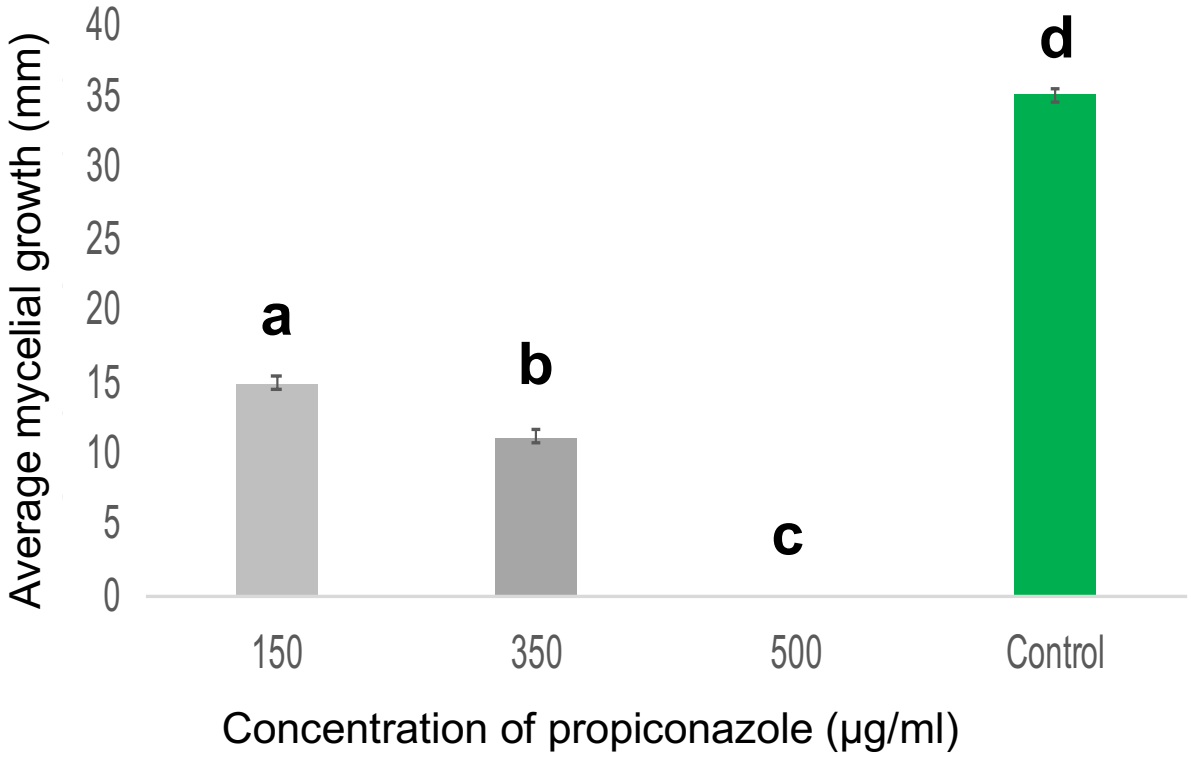


Figure 11: The mean measurements of *Fusarium euwallaceae* mycelia, plated onto different concentrations of ½ PDA, amended with propiconazole, at 3 DAP. The bars indicate the average mycelial growth for eight replicates, as well as the standard error for the data. The statistical analysis revealed Gaussian distribution and an ANOVA of the results showed that there were significant differences between the *F. euwallaceae* average mycelial growth on the amended plates (a, b and c), for all three concentrations, compared to the *F. euwallaceae* plated on unamended, ½ PDA medium (d). This is because the mycelial inhibition differed considerably and significantly between the various concentrations, with complete mycelial inhibition being observed at the highest concentration. Significant differences between the groups are indicated with different letters, where different letters represent significant differences in the average mycelial growth between the two groups being compared.

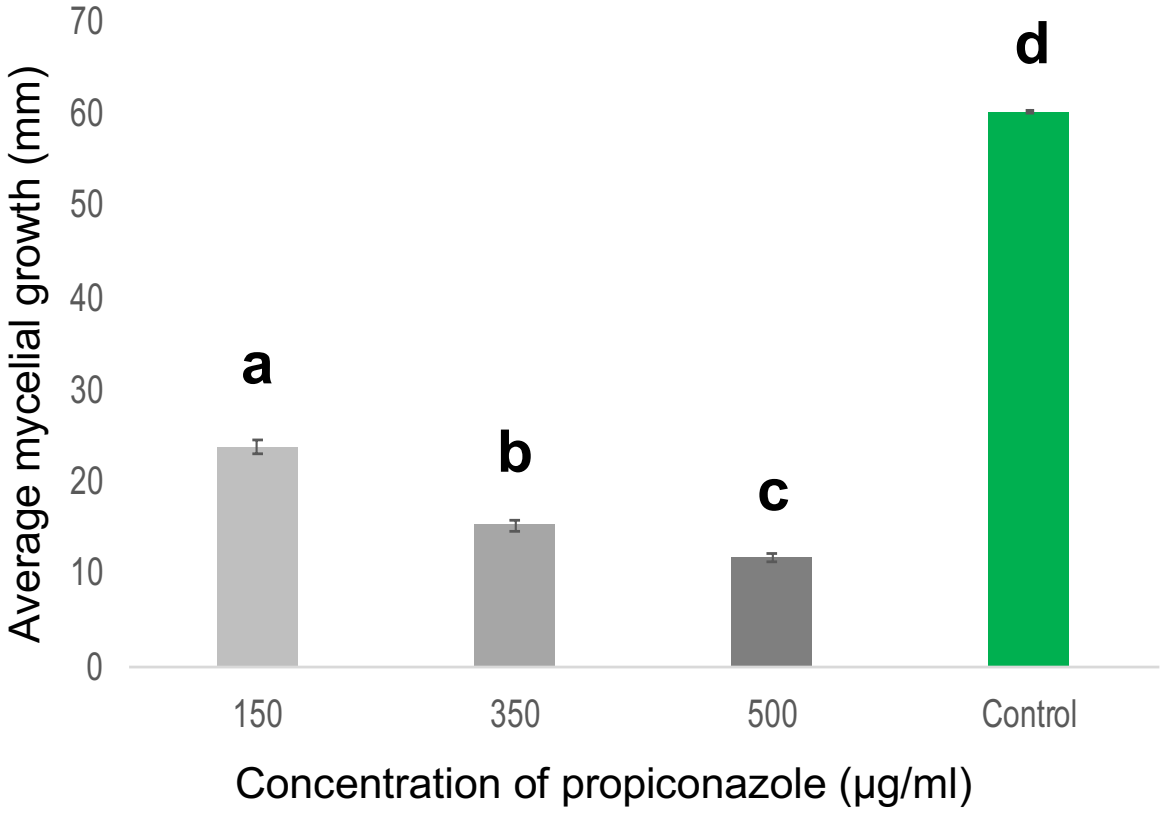


Figure 12: The mean measurements of *Fusarium euwallaceae* mycelia, plated onto ½ PDA, amended with different concentrations of propiconazole, at 6 DAP. The mean and standard error of the mycelial growth are indicated by the bars. An analysis of variance and Tukey's multiple comparisons test, of the normally distributed data, showed significant differences between the average mycelial growth on the amended plates (a, b, c) and control plates (d). The statistical analysis also revealed significant differences between the average mycelial growth of the different concentrations (a, b and c) but to a lesser extent than that of the same comparisons at 3 DAP, due to the growth of mycelia at 500 µg/ml. Bars represented with the same letter are not significantly different at $P < 0.05$. These results indicate a decrease in average mycelial growth that correlates with an increase of concentration but suggests loss of effectivity as time progresses.

***In vitro control assay of Fusarium euwallaceae, using Bacillus* spp.**

Both *Bacillus* spp. culture powder formulations (B-Rus and Mity-Gro) limited the growth of the *F. euwallaceae* mycelia significantly when compared to the control plates. The mycelia of *F. euwallaceae* grew radially outward from the colonised plug, in the centre of the media, until encountering colonies of the *Bacillus* spp. culture powder. At 3 DAP, the *F. euwallaceae* was observed discontinuing further radial growth towards the bacterial colonies and only continued to grow and increase in density around the previously colonised agar plug. The bacteria continued to colonise the surface of the remaining nutrient medium in the days thereafter. Morphological observations of the bacterial colonies and mycelia implied that there was no direct, visible contact between the physical structures of the two organisms, instead, a very thin area of inhibition was observed at the points of expected contact, around the circumference of the *F. euwallaceae* mycelia. Despite other differences, this observation was generally noted for both of the bacterial culture powders that were tested. The main experimental difference between the two culture powders was that the B-Rus culture powder inhibited the growth of *F. euwallaceae* mycelia far more effectively than that of the Mity-Gro culture powder. Images for the results for the B-Rus culture powder assay are shown in **Figure 13** and the mean lesion lengths, standard error and significance thereof are illustrated in **Figure 14** and **Figure 15.** Images for the results for the Mity-Gro culture powder assay are shown in **Figure 16** and the quantitative and statistical analysis of the mycelial growth are illustrated in **Figure 17** and **Figure 18.** The quantitative data and statistical analysis of the results from these assays are shown in **Tables 14 – 21**, in the supplementary information.

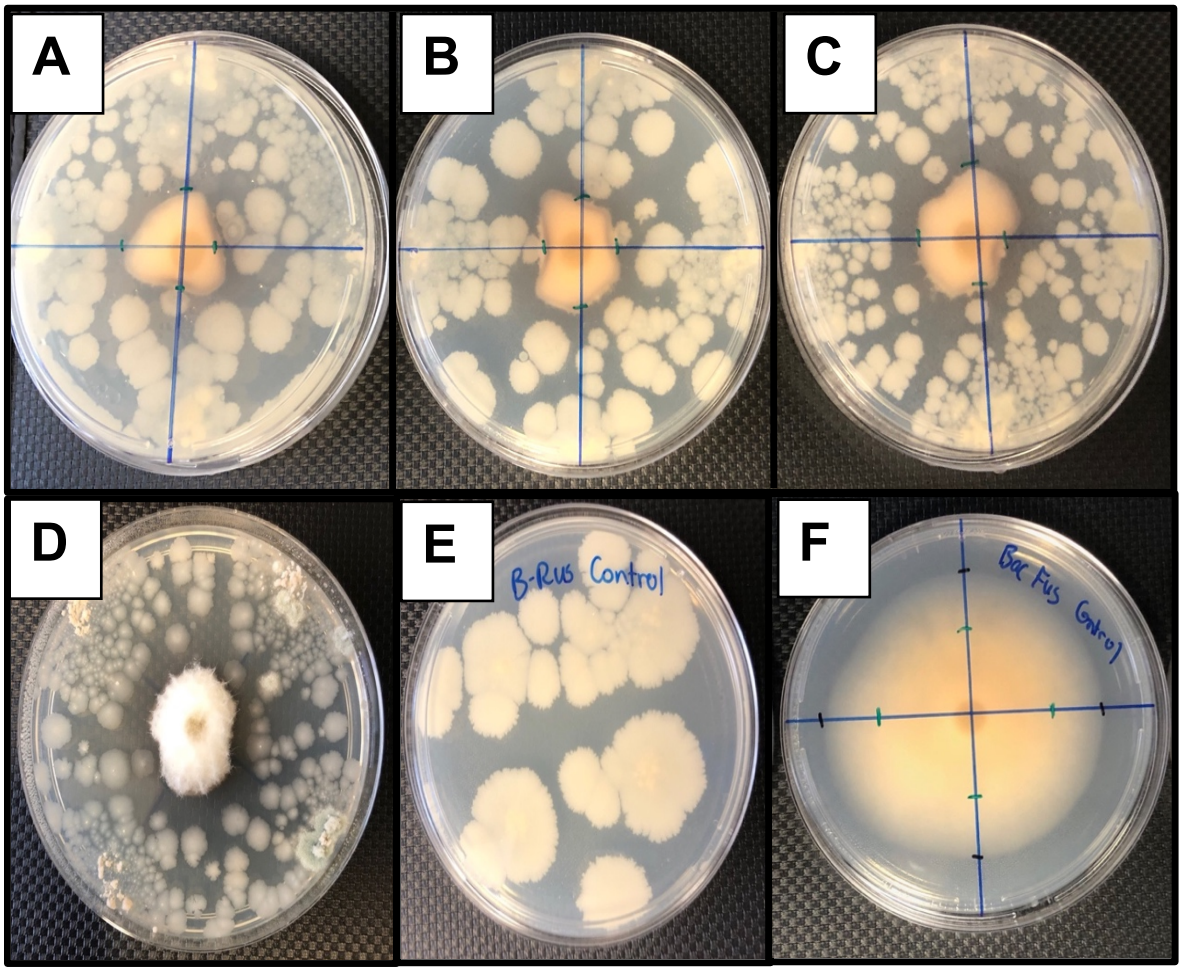


Figure 13: The morphology of the *Fusarium euwallaceae* culture, plated on ½ PDA nutrient medium, together with the B-Rus, *Bacillus* spp. culture powder. The images labelled A – C indicate the plates at 3 DAP. The *F. euwallaceae* mycelia did not further expand its margins, even at 6 DAP (not shown here). Image D shows the *F. euwallaceae* mycelia clumped around the agar plug and the accompanying bacterial colonies. Image E shows a control plate containing only *Bacillus* spp. colonies, to ensure successful activation of the culture powder. Image F shows the average mycelial growth of *F. euwallaceae,* plated on unamended ½ PDA medium and its mean mycelial growth was compared to the dual-plate cultures, at both 3 DAP and 6 DAP.

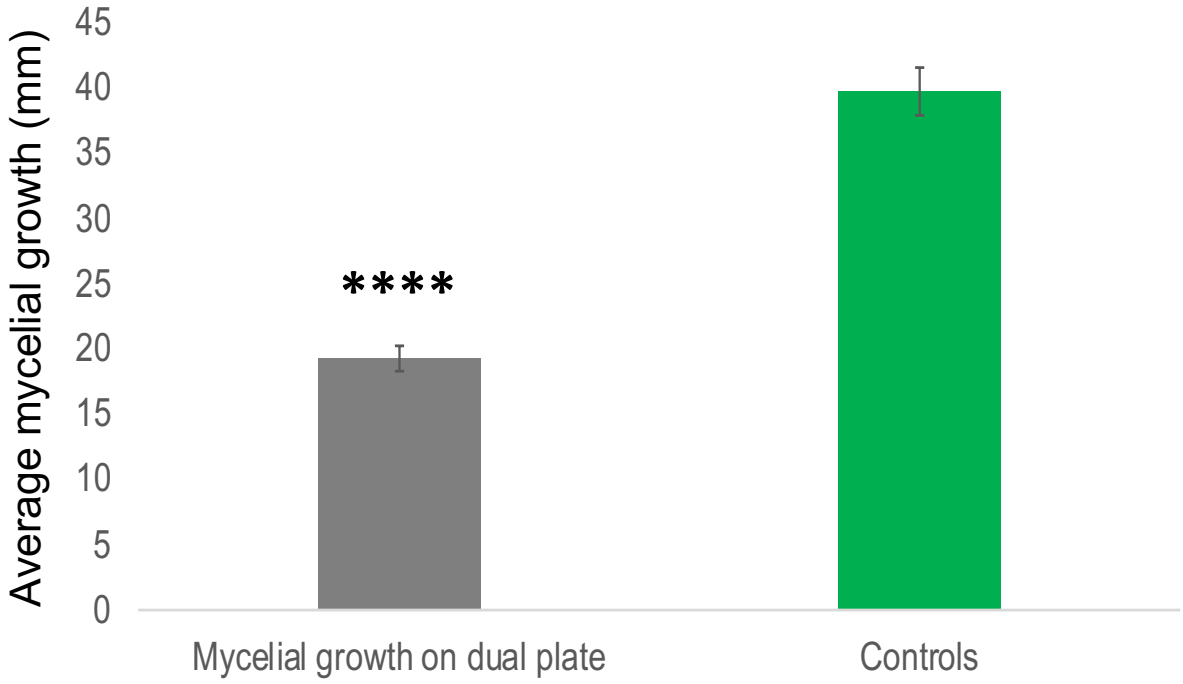


Figure 14: The mean measurements of *Fusarium euwallaceae* mycelia, dual-cultured on ½ PDA, along with B-Rus, *Bacillus* spp. culture powder, at 3 DAP. The average *F. euwallaceae* mycelial growth of ten replicates is indicated by the grey bar, accompanied by the standard error. The data was normally distributed and a one-way-ANOVA with Tukey's multiple comparisons test revealed a significant difference between the average mycelial growth of *F. euwallaceae* on the dual-plates, compared to the *F. euwallaceae* plated on sterile, ½ PDA medium.

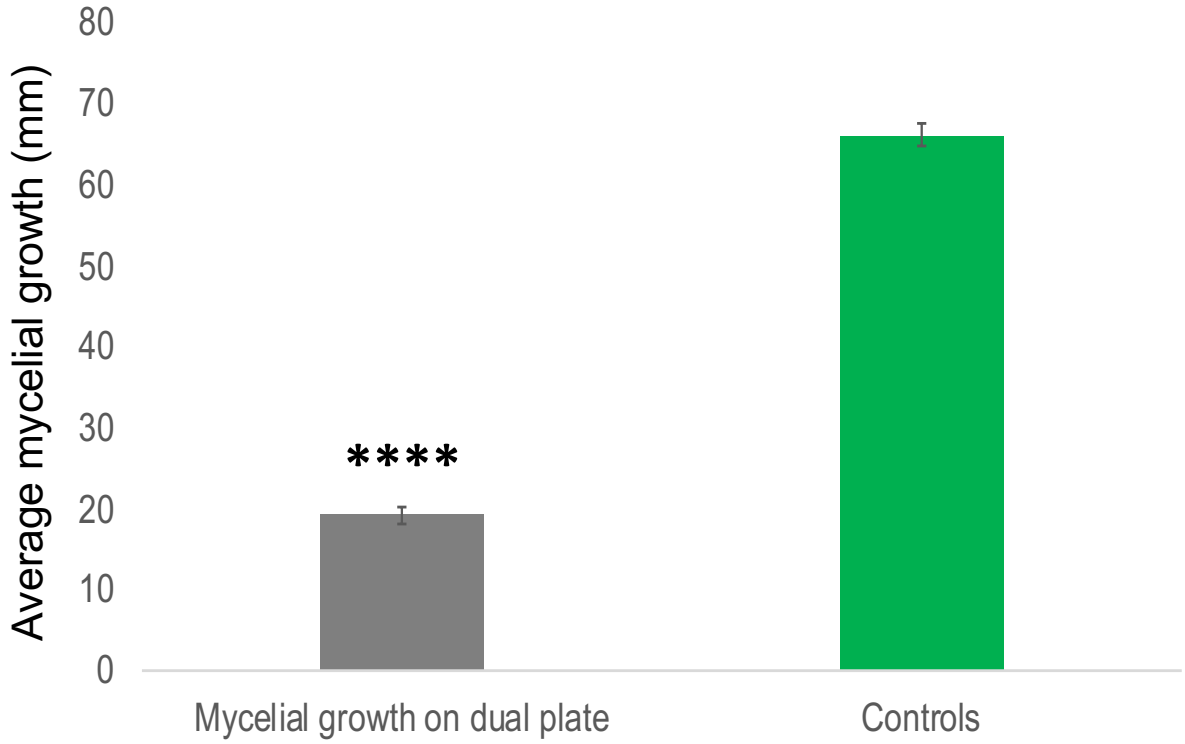


Figure 15: The mean measurements of *Fusarium euwallaceae* mycelia, dual-cultured with B-Rus, *Bacillus* spp. culture powder, at 6 DAP. The data followed non-Gaussian distribution and a non-parametric Kraskal-Willis test and Dunn's multiple comparisons test revealed a significant difference between the average mycelial growth of *F. euwallaceae* on the dual-plates, compared to the *F. euwallaceae* plated on sterile, ½ PDA medium at 6 DAP. The grey bar indicates the mean and standard error of the mycelial growth of the ten replicates, as well as the significant difference between the dual-plates and control plates.

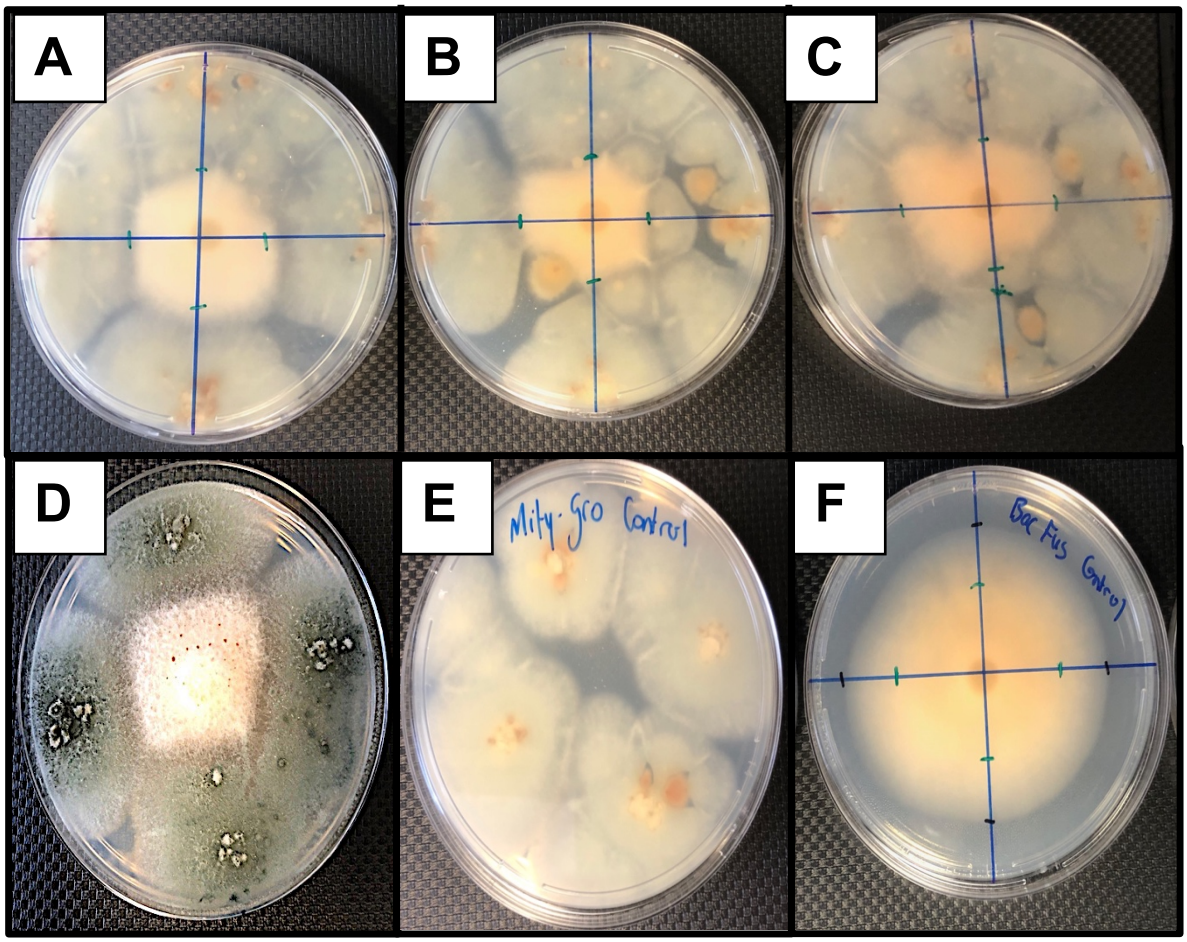


Figure 16: The culture morphology of *Fusarium euwallaceae*, plated onto nutrient medium, together with the Mity-Gro, *Bacillus* spp. culture powder. The images labelled A – C indicate the plates at 3 DAP. The *F. euwallaceae* mycelia did not further expand its margins, even at 6 DAP (not shown here). Image D shows the clumped *F. euwallaceae* mycelium and the accompanying bacterial colonies. Image E shows a control plate containing only *Bacillus* spp. colonies, to ensure successful activation of the culture powder. Image F shows the average mycelial growth of *F. euwallaceae,* plated on ½ PDA medium. The mean mycelial growth of the control plate was compared to the dual-plate cultures, at 3 DAP and 6 DAP, to determine if the significance and degree of difference.

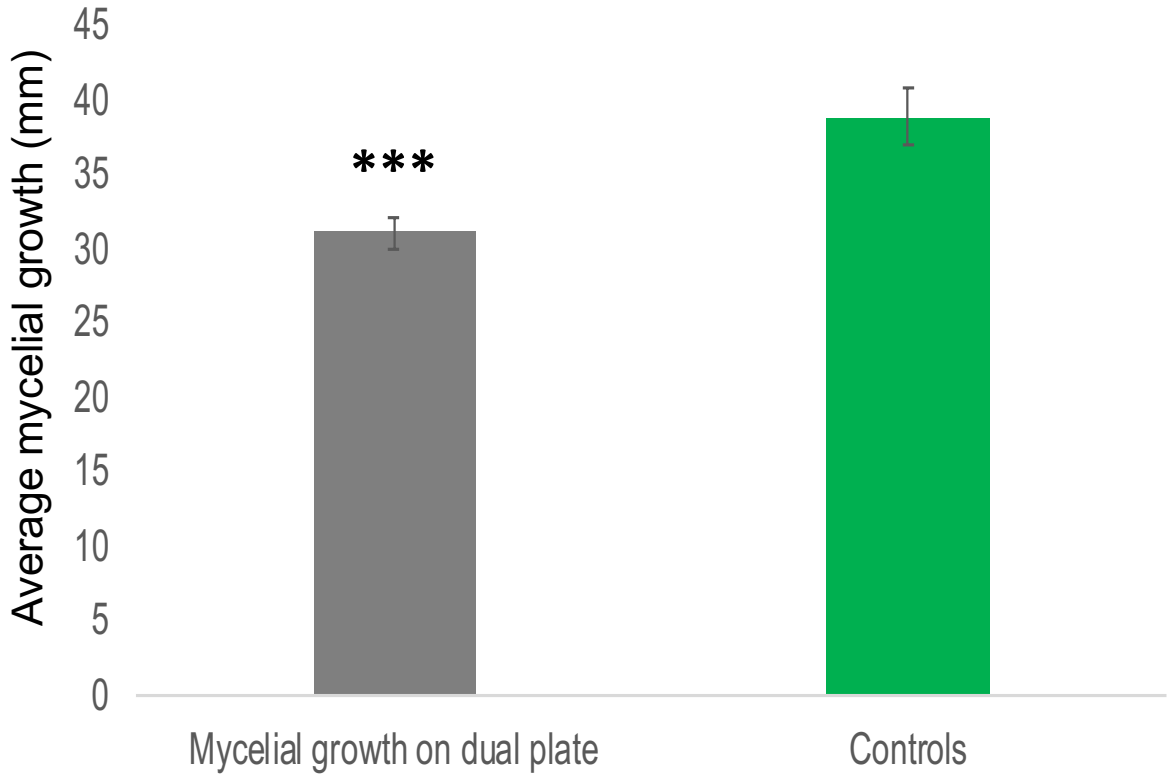


Figure 17: The mean measurements of *Fusarium euwallaceae* mycelia, dual-cultured with Mity-Gro, *Bacillus* spp. culture powder, at 3 DAP. A one-way-ANOVA with Tukey's multiple comparisons test, revealed a significant difference between the average mycelial growth of *F. euwallaceae* on the dual-plates, compared to the *F. euwallaceae* plated on sterile, ½ PDA medium, indicated by asterisks. The data was normally distributed and the mean and standard error of the mycelial growth of the ten replicates and controls are indicated by the bars. At 3 DAP, the average mycelial growth for *F. euwallaceae,* cultured with Mity-Gro, was larger than that observed for the B-Rus dual-culture plates.

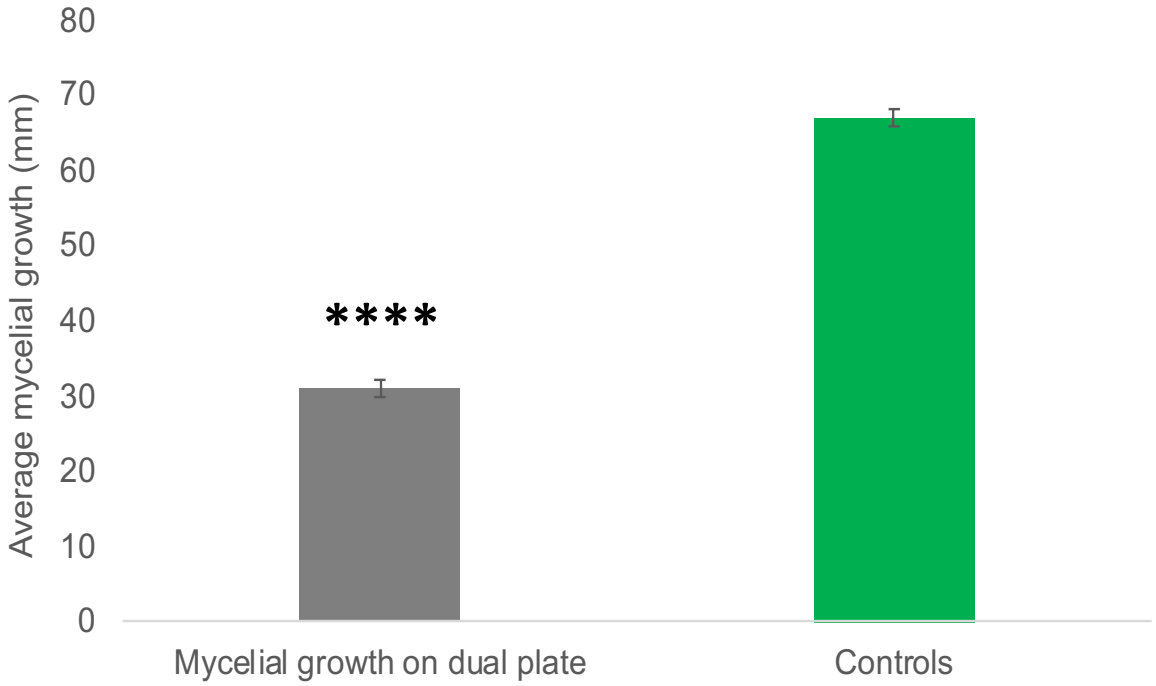


Figure 18: The mean measurements of *Fusarium euwallaceae* mycelia, dual-cultured onto ½ PDA, with Mity Gro, *Bacillus* spp. culture powder, at 6 DAP. The mean of the ten replicates, indicated by the grey bar, shows how the average mycelial growth of *F. euwallaceae* remained the same after 3 days. A one-way-ANOVA showed an increase in the significance of the difference of the data, from 3 DAP, indicated by four asterisks, compared to the three asterisks at 3 DAP.

***In vitro control assay against Fusarium euwallaceae, using Trichoderma* spp.**

The *Trichoderma* spp. that was tested limited the growth of the *F. euwallaceae* mycelia significantly when compared to the mycelia of *F. euwallaceae* on the control plates. *F. euwallaceae* grew, radially outward from the colonised plug, on one side of the Petri-plate, until encountering the *Trichoderma* mycelia, actively growing from the other side of the Petri-plate. Similarly to that observed from the bacterial assay, at 3 DAP, the *F. euwallaceae* was observed discontinuing further radial growth towards the *Trichoderma* mycelia and only continued to grow and increase in density around the colonised agar plug. The *Trichoderma* mycelia continued to colonise the surface of the remaining nutrient medium in the days thereafter. Morphological observations of the *Trichoderma* mycelia and *F. euwallaceae* mycelia implied that there was visible contact between the physical structures of two organisms, around the circumference of the *F. euwallaceae* mycelium. The outermost growth borders of both fungi were maintained and did not proceed to grow into one another's mycelia. Because this study was only interested in the growth of the *F. euwallaceae* mycelia, no measurements were taken for the *Trichoderma* mycelia. Images for the results for the *Trichoderma* spp. assay are shown in **Figure 19** and the mean lesion lengths, the standard error and significance thereof are graphically illustrated in **Figure 20 and Figure 21.** The quantitative data and statistical analysis of the results from these assays are shown in **Tables 22 – 25**, in the supplementary information.

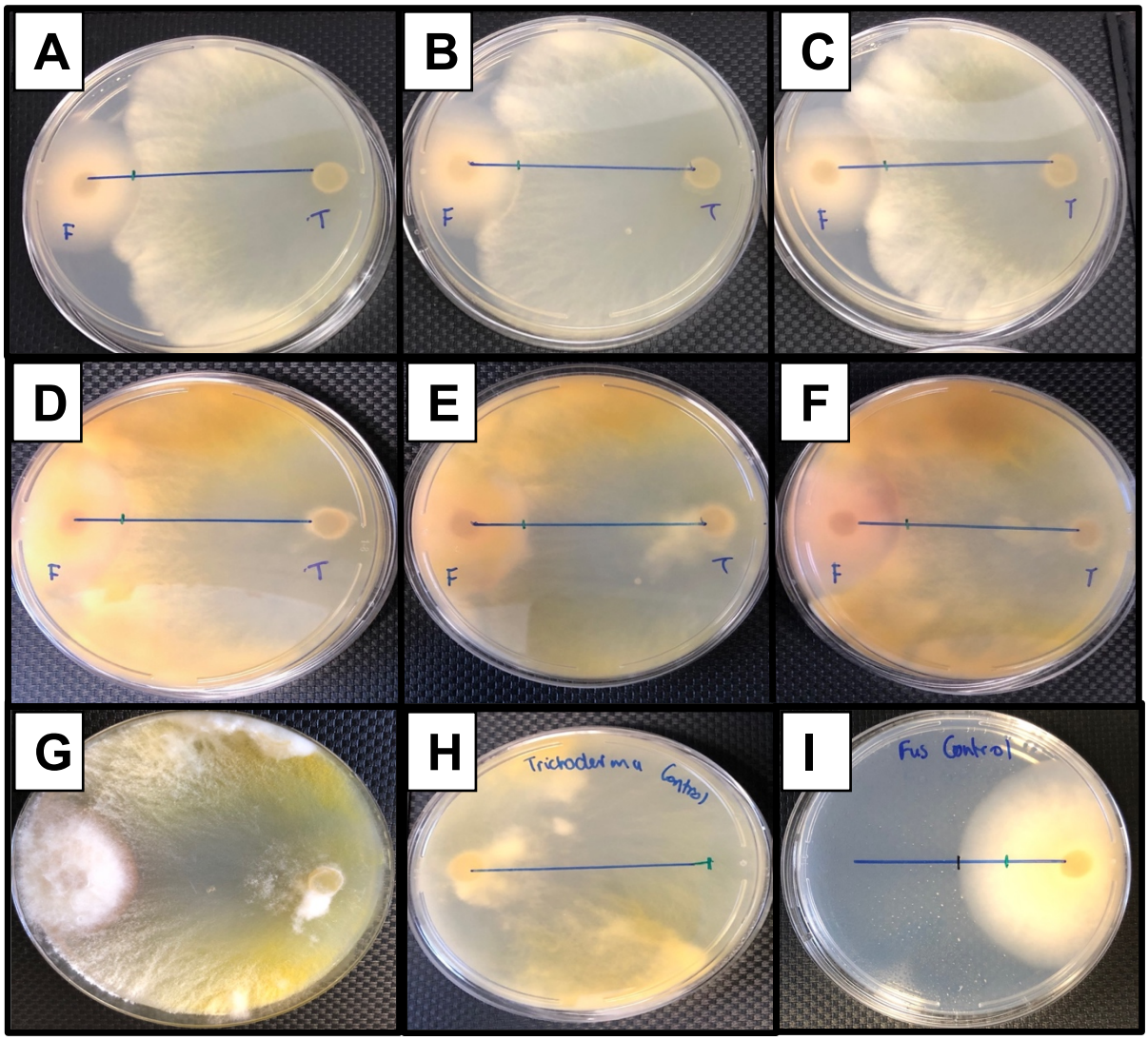


Figure 19: The culture morphology of *Fusarium euwallaceae*, plated on nutrient medium, together with the *Trichoderma* spp. colonised agar plugs. The images labelled A – C indicate the plates at 3 DAP (days after plating) and the images labelled D – F indicate the plates at 6 DAP. The *F. euwallaceae* mycelia did not further expand its margins, even at 6 DAP. Image G shows clumped *F. euwallaceae* mycelia (on the left of the plate) and accompanying *Trichoderma* mycelia (on the right of the plate). Image E shows a control plate containing only *Trichoderma* mycelia, to ensure that the isolate was actively growing. Image F shows the average mycelial growth of *F. euwallaceae,* plated on ½ PDA medium and its mean mycelial growth was compared to the dual-plate cultures, at both 3 DAP and 6 DAP.

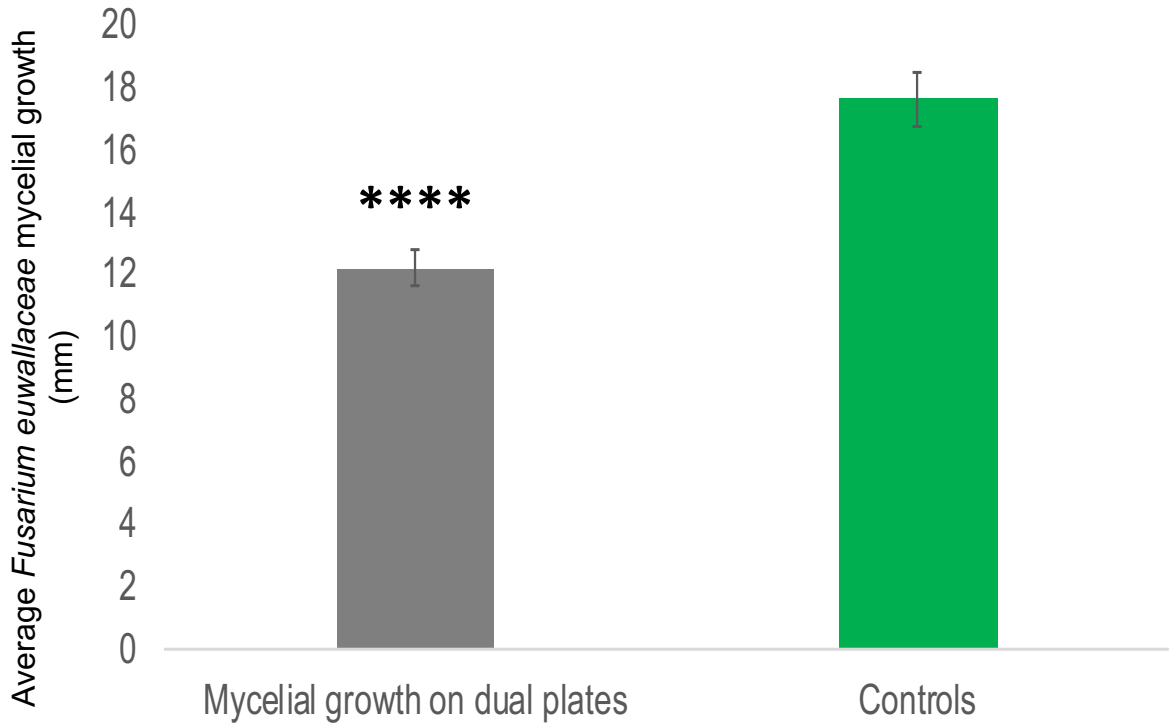


Figure 20: The mean *Fusarium euwallaceae* mycelial measurements, dual-cultured, with *Trichoderma* spp., at 3 DAP. The grey bar indicates the mean of the mycelial growth of the ten replicates, as well as the significant difference between the dual-plates and control plates, indicated by asterisks (*). More asterisks indicate higher degrees of significance. The data followed Gaussian distribution and a one-way-ANOVA with Tukey's multiple comparisons test revealed a significant difference between the average mycelial growth of *F. euwallaceae* on the dual-plates, compared to the *F. euwallaceae* plated on sterile, ½ PDA medium.

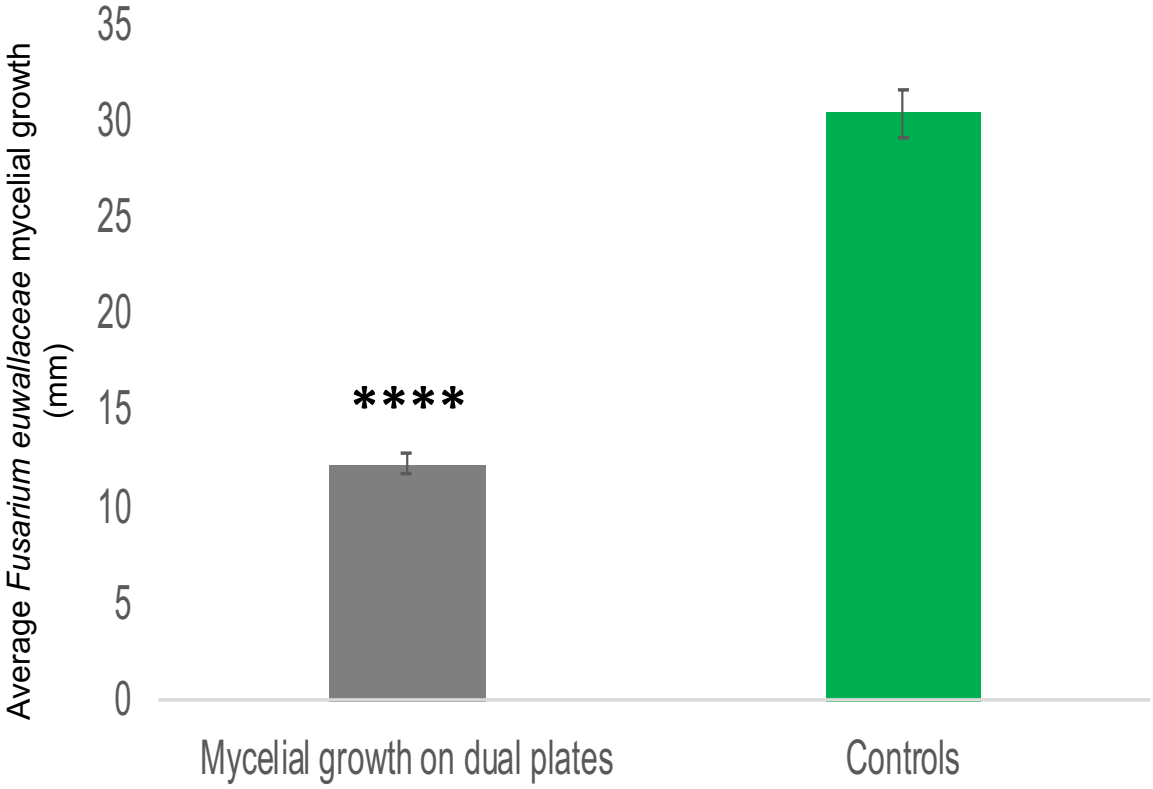


Figure 21: The average measurements of *Fusarium euwallaceae* mycelia, dual-cultured onto ½ PDA, with *Trichoderma* spp., at 6 DAP. This data shows how the average mycelial growth of *F. euwallaceae* remained the same after 3 days and a statistical analysis showed that the data followed normal distribution and a multiple comparison test revealed a significant difference between the average mycelial growth of *F. euwallaceae* on the dual-plates, compared to the control plates.

DISCUSSION

The research conducted in this chapter included several notable findings. Firstly, this study showed that Edranol and Ettinger, two previously untested green-skin avocado cultivars were both susceptible to FD. This was observed while investigating potential differences in susceptibility among the two commercial avocado fruiting cultivars, in response to infection by the PSHB fungal associates. However, accurate conclusions with regards to differences in susceptibility could not be assessed, as the plantlets differed in age, meaning inferences could not be made based on observed differences in susceptibility, between the cultivars.

Nonetheless, one of the findings of this pathogenicity trial included the observation of the standalone pathogenicity of one of the other fungal symbionts of the PSHB, other than *F. euwallaceae* – namely *G. euwallaceae*, on plantlets from both avocado cultivars (Edranol and Ettinger). This is noteworthy as *G. euwallaceae* was previously thought to be involved only as a suitable food source for the PSHB, specifically the larvae, to segregate the food sources from the adults from that of the developing larvae (Freeman *et al.*, 2016). In this study, *G. euwallaceae* caused disease symptoms on avocado plantlets and statistical analysis of the associated lesions was proven to be significantly larger than the lesions on the control plantlets. The lesions from the *G. euwallaceae* only inoculation caused lesions that were significantly smaller and less severe than that of the lesions caused by *F. euwallaceae,* proving that *F. euwallaceae* is more pathogenic, to avocado.

Another observation from the pathogenicity trial, that substantiates the potential pathogenicity of *G. euwallaceae,* and the evolutionary sophistication between the

PSHB and its fungal associates, was that the effect of a co-inoculation of *G. euwallaceae* and *F. euwallaceae* yielded lesions significantly larger than the sum of both the lesions caused by *F. euwallaceae* and *G. euwallaceae* when inoculated on their own. This result correlates directly to field conditions, as infestation by the PSHB will likely inoculate more than one of its mycangial symbionts into host tissue (Lynch *et al.*, 2016) and the consequential disease (FD) is a result of the action by more than *F. euwallaceae* alone. This previously unapparent conclusion means that the individual interactions between the mycangial fungi, harbored by the PSHB, are more complex than initially anticipated (Freeman *et al.*, 2012) and all three need to be studied, for their combined pathogenesis and virulence.

An improvement, to the pathogenicity trial that was conducted in this study, would be to include the other fungal symbiont of the PSHB - *Paracremonium pembeum,* for further co-inoculation studies. To date, *P. pembeum,* has yet to be isolated, from South Africa. The presence of *P. pembeum* has been shown to be reduced in abundance, when compared to the other PSHB symbionts and has shown almost exclusive association to the nutrition of immature beetles and larvae (Freeman *et al.*, 2016), thereby decreasing chances for its isolation from already symptomatic plant material. Yet, results from this study suggest that another PSHB symbiont, other than *F. euwallaceae,* has the ability to cause disease when inoculated on its own and further suggests the potential for synergistic pathogenicity of the PSHB fungal associates.

Possible differences in susceptibility among commercial avocado fruiting cultivars, in response to infestation by the PSHB, have been suggested by Jones & Paine (2017). In their study, they found the Fuerte, Gwen, and Bacon avocado cultivars indicated lower attack rates by the PSHB, as well as lower rates of gallery formation, when compared to some of the other cultivars that were tested, such as Hass, Pinkerton,

Reed, Gem and Zutano (Jones & Paine, 2017). Their study investigated only attack rates by the PSHB and rates of gallery formation, therefore there is an equal necessity to evaluate differences in susceptibility of these cultivars, based on their response to infection by the fungal associates of the PSHB. Future studies regarding this would involve inoculations and co-inoculations of more plantlets, from different cultivars, at the same age, such that conclusions regarding differences in susceptibility could be made more accurately and definitely.

In addition to selecting for plant varieties, with resistance or higher tolerance, to FD, another approach to control the disease is through the direct application of chemical and biological control agents to limit the growth of pathogen/pathogens responsible for the disease (Nene & Thapliyal, 1993, Castrillo *et al.*, 2016). This study screened the effectivity and longevity of several fungicides and biological control agents that were noted, by literature (Jones *et al.*, 2017, Guevara-Avendaño *et al.*, 2018), for promising prospects to control *F. euwallaceae*. All the agents that were tested indicated promising *in vitro* results, for the control of *F. euwallaceae,* even at their lowest concentrations. It is noteworthy that for two of the four fungicides (fluazinam and propiconazole), actively growing *F. euwallaceae* mycelia continued to grow and increase in diameter as time progressed, alluding to their limitations as short-term-only control measures. However, for the other two fungicides (metconazole and tebuconazole) mycelial inhibition persisted through the assay, suggesting that they retained their efficacy, but high concentrations and differences in toxicity, in field conditions, may mean that the other fungicides should also be considered. Often, high concentrations and over-frequent application of a single chemical agent can compromise the biological integrity of the ecosystem or plants being treated (Sarwar,

2015), this being especially true for applications for commercially active fruit trees, such as avocado.

An important observation from this study concludes that *in vitro* control assays, of chemical agents, should be considered with the pairwise observations generated by field-trial experiments. As observed in the results generated by this study, it is obvious that *in vitro* screening for chemical agents is far more sensitive, easily tested and can be adjusted more accurately. However, complications may arise when seeking to apply said chemicals, in field conditions, in that underestimations for the necessary concentrations may occur. This is illustrated when comparing the results generated by this study, with that observed in the *in planta* assay, reported on by Mayorquin *et al.* (2018). When comparing the *in planta* EC50, described by Mayorquin *et al.* (2018), it is clear that the same concentrations applied, *in vitro*, yielded far higher levels of inhibition, thereby alluding to the *in vitro* underestimations that may occur. Nonetheless, as a starting point, it is always necessary to determine approximate effective concentrations, *in vitro,* before attempting to conduct a field- or *in planta* trial, especially for compounds which have not been previously tested against the organism of interest, in previous studies (Sharma & Kumar, 2009).

In addition to applying chemical agents, farming systems have started to employ the use of environmentally-friendly, biological agents, as control means for insect-vectors (Nahrung *et al.*, 2016) of agriculturally important pathogens, such as ambrosia beetles (Carrillo *et al.*, 2015). The application of biological control agents faces many limitations and uncertainties and is often met with doubt and bias, as the use of a live organism, to control another organism, may have unpredictable and unforeseen implications in the future (Louda & Stiling, 2004). Even so, many agriculturally

important vector-borne diseases have been controlled in the past (Bedding & Akhurst, 1974).

In this study, commercially available biological controls were tested for their ability to reduce the growth of *F. euwallaceae* mycelia, *in vitro,* by means of dual-culture assays. Results of which indicated that both the *Trichoderma* spp. and *Bacillus* spp. biological control organisms were able to significantly limit the growth of *F. euwallaceae* mycelia when compared to control plates containing only *F. euwallaceae*. In addition, the rate of mycelial growth/colony formation, for both groups of biological control organisms, was much higher than the rate of mycelial growth displayed by the *F. euwallaceae* mycelia. This observed biological control results either from competition for nutrients/space or as a direct result interaction between the biological control agent and the pathogen itself, through mycoparasitism or the synthesis of toxins or hydrolytic enzymes (Fravel, 1988). The physicochemical conditions, that these organisms subjected to, often results in unpredictable modes of action (Benítez *et al.*, 2004).

One observation, in this study, that was noticeably different from the interaction seen during the *Trichoderma* spp. and *F. euwallaceae* assay was that the bacterial *Bacillus* spp. colonies and the *F. euwallaceae* mycelia did not appear to come into physical contact with one another. Instead, what appeared to be a clearance zone, absent of either organism's growth, was observed where they would otherwise come into contact. This suggests the out-competition of *F. euwallaceae* by the *Trichoderma* spp. and conversely the production of bacteriostatic or fungistatic compounds by *F. euwallaceae* and *Bacillus* spp., respectively, both resulting in biological control of *F. euwallaceae* (Benítez *et al.*, 2004, Ongena & Jacques, 2008).

Many members from the *Bacillus* genus are considered safe micro-organisms, that often exist as epiphytes in healthy plants and have shown promising potential as biological control organisms, effective at inhibiting the growth of a wide variety of fungi (Cazorla *et al.*, 2007). Bacteria from this genus synthesise a considerable amount of different compounds and antimicrobials, such as antibiotics and lipopeptides (Ongena & Jacques, 2008) that have been shown to be effective against numerous phytopathogens (Leifert *et al.*, 1995). In contrast, most *Trichoderma* spp., used as biological control agents, are effective as they can outcompete many fungal phytopathogens, by producing highly-efficient siderophores that rapidly chelate iron and stop the growth of other fungi (Benítez *et al.*, 2004). Many *Trichoderma* spp. are also suitable as *in planta* biological control agents as they are known to exert positive effects on plants leading to increased plant growth and the stimulation of plant-defense mechanisms (Benítez *et al.*, 2004). In a process known as biofertilisation, roots are colonised by *Trichoderma* spp., thereby leading to enhanced root growth and development and increased uptake in nutrients (Kubicek *et al.*, 2001).

The biological control organisms and chemical agents used in this study can, therefore, be considered alongside the use of other control strategies, to treat infected plants and to limit the further damage caused by the PSHB and its associated fungal pathogens. These chemical control agents could theoretically be used in field-based trials, where they would be applied in simultaneously, in various ways. Fluazinam is neither systemic nor curative, instead, it is a protectant fungicide and would likely be effective in the form of a trunk spray (Hodel, 2017), along with a protectant insecticide, to reduce the chance of infection and inhibiting the germination of fungal spores (Jones *et al.*, 2017). The three triazole agents that were tested are systemic fungicides and sterol biosynthesis inhibitors, therefore a trunk injection would (Byrne *et al.*, 2012,

Hodel, 2017) would be aimed at the internal and systemic inhibition of the formation of fungal cell membranes. The biological controls are usually proposed as soil enhancers (Cernansky, 2015), to increase the overall health and fitness of valuable plants that have been confirmed to be affected with FD. The long-term prophylactic abilities, of these control agents would likely rely on frequent and combined re-application of the effective agent/agents (Hodel, 2017). This approach should be accompanied by considerations to limit or control the insect vector through responsible management of infested plant material through pruning and incineration of infested trees and fine-scale wood chipping and subsequent exposure to sunlight in the form of solarisation (Jones & Paine, 2015), as a more economical and sustainable solution. However, controlling ambrosia beetle populations and the pathogens that they spread, that occupy a wide geographic distribution, can prove particularly difficult and most available control strategies do not confer protection to trees in areas that are not routinely monitored for the presence of ambrosia beetle damage (Ploetz *et al.*, 2013).

Historical evidence of damage caused by ambrosia beetles, and other agricultural pests, suggests that an integrated combination of management and preventative approaches must be applied to combat invasive pests (Shea *et al.*, 2000) and their associated fungal partners, in the form of proper monitoring and management of infested plant material, resistant/tolerant cultivar selection and/or the use of chemical and biological control agents.

CONCLUSION

The recent introduction of the PSHB, into South Africa (Paap *et al.*, 2018), has acted as a disrupting factor to reveal a direct effect of globalisation and climate change. Ambrosia beetles, such as the PSHB, are almost always considered as un-invasive and vitally important members in their natural ecosystems (Borden, 1988), where they usually fill important environmental niches and/or their numbers are suppressed due to the presence of a natural enemy (Frank & Sadof, 2011). When these organisms are introduced into areas that they have not been present before, various forms of environmental impacts occur (Carter, 1962). In the case of the PSHB, international perspectives have alluded to the threat, posed by this pest complex, particularly to the avocado fruit crop (Mendel *et al.*, 2012, Eskalen *et al.*, 2013, Freeman *et al.*, 2013, van den Berg *et al.*, 2019). The threat posed by the newly introduced ambrosia beetle pest complex, in South Africa, is one that must be regarded as an issue of importance.

It is therefore necessary that this pest complex must be monitored and that management schemes are continuously revised. Basic research and primary investigations into the underlying aspects of the disease lifestyle can highlight aspects that may be targeted for the control of the disease. It is a social responsibility to create awareness of this problem and that environmentally-friendly and long-term control strategies be created, by those who have the ability to do so and from a scientific perspective, it means that the effectivity, cost and feasibility of the proposed control strategies should be thoroughly and responsibly tested and scrutinised.

Basic research, such as the investigations from this and the other studies (Cernansky, 2015, Jones *et al.*, 2017, Jones & Paine, 2017), suggests that an integrated approach of various methods is necessary to combat this pest complex effectively. This includes

the use of resistant crop cultivars, the use of non-harmful, synthetic chemicals and the use of naturally occurring biological organisms. The nature of the research conducted in this, and the previous, study indicates the substantial complexity of the FD disease lifecycle and the lack of overall scientific knowledge and awareness among the general public. Such a lack of scientific evidence and scarcity of skill to study this phenomenon means that the potential damage from this invasive pest complex could be vast, if not properly investigated and studied accordingly.

REFERENCES

Atkinson TH, Foltz JL, Wilkinson RC & Mizell RF (1988) Granulate ambrosia beetle, *Xylosandrus crassiusculus* (Motschulsky) (Insecta: Coleoptera: Curculionidae: Scolytinae). *Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Entomology Circular* **310**.

Bedding R & Akhurst R (1974) Use of the nematode *Deladenus siridicola* in the biological control of *Sirex noctilio* in Australia. *Australian Journal of Entomology* **13**: 129-135.

Benítez T, Rincón AM, Limón MC & Codon AC (2004) Biocontrol mechanisms of *Trichoderma* strains. *International Microbiology* **7**: 249-260.

Borden JH (1988) The striped ambrosia beetle. *Dynamics of Forest Insect Populations* 579-596. Springer.

Byrne FJ, Urena AA, Robinson LJ, Krieger RI, Doccola J & Morse JG (2012) Evaluation of neonicotinoid, organophosphate and avermectin trunk injections for the management of avocado thrips in California avocado groves. *Pest Management Science* **68**: 811-817.

Carrillo D, Dunlap C, Avery P, Navarrete J, Duncan R, Jackson M, Behle R, Cave R, Crane J & Rooney A (2015) Entomopathogenic fungi as biological control agents for the vector of the laurel wilt disease, the redbay ambrosia beetle, *Xyleborus glabratus* (Coleoptera: Curculionidae). *Biological Control* **81**: 44-50.

Carter W (1962) Insects in relation to plant disease. *Insects in relation to plant disease*. New York, N.Y. & London, Interscience Publications

Castrillo LA, Griggs MH & Vandenberg JD (2013) Granulate ambrosia beetle, *Xylosandrus crassiusculus* (Coleoptera: Curculionidae), survival and brood production following exposure to entomopathogenic and mycoparasitic fungi. *Biological Control* **67**: 220-226.

Castrillo LA, Griggs MH & Vandenberg JD (2016) Competition between biological control fungi and fungal symbionts of ambrosia beetles *Xylosandrus crassiusculus* and *X. germanus* (Coleoptera: Curculionidae): mycelial interactions and impact on beetle brood production. *Biological Control* **103**: 138-146.

Cazorla F, Romero D, Pérez-García A, Lugtenberg B, Vicente Ad & Bloemberg G (2007) Isolation and characterization of antagonistic *Bacillus subtilis* strains from the avocado rhizoplane displaying biocontrol activity. *Journal of Applied Microbiology* **103**: 1950-1959.

Cernansky R (2015) Agriculture: State-of-the-art soil. *Nature News* **517**: 258.

Eskalen A, Stouthamer R, Lynch SC, Rugman-Jones PF, Twizeyimana M, Gonzalez A & Thibault T (2013) Host range of Fusarium dieback and its ambrosia beetle (Coleoptera: Scolytinae) vector in southern California. *Plant Disease* **97**: 938-951.

Evans EA, Crane J, Hodges A & Osborne JL (2010) Potential economic impact of laurel wilt disease on the Florida avocado industry. *HortTechnology* **20**: 234-238.

Frank SD & Sadof CS (2011) Reducing insecticide volume and nontarget effects of ambrosia beetle management in nurseries. *Journal of Economic Entomology* **104**: 1960-1968.

Fravel DR (1988) Role of antibiosis in the biocontrol of plant diseases. *Annual Review of Phytopathology* **26**: 75-91.

Freeman S, Protasov A, Sharon M, Mohotti K, Eliyahu M, Okon-Levy N, Maymon M & Mendel Z (2012) Obligate feed requirement of *Fusarium* sp. nov., an avocado wilting agent, by the ambrosia beetle *Euwallacea* aff. fornicata. *Symbiosis* **58**: 245-251.

Freeman S, Sharon M, Maymon M, Mendel Z, Protasov A, Aoki T, Eskalen A & O'Donnell K (2013) *Fusarium euwallaceae* sp. nov.—a symbiotic fungus of *Euwallacea* sp., an invasive ambrosia beetle in Israel and California. *Mycologia* **105**: 1595-1606.

Freeman S, Sharon M, Dori-Bachash M, Maymon M, Belausov E, Maoz Y, Margalit O, Protasov A & Mendel Z (2016) Symbiotic association of three fungal species throughout the life cycle of the ambrosia beetle *Euwallacea* nr. *fornicatus. Symbiosis* **68**: 115-128.

Guevara-Avendaño E, Carrillo JD, Ndinga-Muniania C, Moreno K, Méndez-Bravo A, Guerrero-Analco JA, Eskalen A & Reverchon F (2018) Antifungal activity of avocado rhizobacteria against *Fusarium euwallaceae* and *Graphium* spp., associated with

Euwallacea spp. nr. *fornicatus*, and *Phytophthora cinnamomi*. *Antonie van Leeuwenhoek* **111**: 563-572.

Hodel DR (2017) Polyphagous Shot Hole Borer and Fusarium Dieback Disease on Palms. *Palm Arbor* **1**: 1-17.

Hulcr J & Stelinski LL (2017) The ambrosia symbiosis: from evolutionary ecology to practical management. *Annual Review of Entomology* **62**: 285-303.

Jones M & Paine TD (2017) Differences among avocado cultivars in susceptibility to polyphagous shot hole borer *(Euwallacea* spec.). *Entomologia Experimentalis et Applicata* **163**: 296-304.

Jones ME, Kabashima J, Eskalen A, Dimson M, Mayorquin JS, Carrillo JD, Hanlon CC & Paine TD (2017) Evaluations of insecticides and fungicides for reducing attack rates of a new invasive ambrosia beetle (*Euwallacea* sp., Coleoptera: Curculionidae: Scolytinae) in infested landscape trees in California. *Journal of Economic Entomology* **110**: 1611-1618.

Jones ME & Paine TD (2015) Effect of chipping and solarization on emergence and boring activity of a recently introduced ambrosia beetle (*Euwallacea* sp., Coleoptera: Curculionidae: Scolytinae) in southern California. *Journal of Economic Entomology* **108**: 1852-1859.

Jones ME & Paine TD (2018) Potential pesticides for control of a recently introduced ambrosia beetle (*Euwallacea* sp.) in southern California. *Journal of Pest Science* **91**: 237-246.

Kubicek C, Mach R, Peterbauer C & Lorito M (2001) *Trichoderma*: from genes to biocontrol. *Journal of Plant Pathology* 11-23.

Leifert C, Li H, Chidburee S, Hampson S, Workman S, Sigee D, Epton H & Harbour A (1995) Antibiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. *Journal of Applied Bacteriology* **78**: 97-108.

Louda SM & Stiling P (2004) The double-edged sword of biological control in conservation and restoration. *Conservation Biology* **18**: 50-53.

Lynch SC, Twizeyimana M, Mayorquin JS, Wang DH, Na F, Kayim M, Kasson MT, Thu PQ, Bateman C & Rugman-Jones P (2016) Identification, pathogenicity and abundance of *Paracremonium pembeum* sp. nov. and *Graphium euwallaceae* sp. nov.—two newly discovered mycangial associates of the polyphagous shot hole borer *(Euwallacea* sp.) in California. *Mycologia* **108**: 313-329.

Mayorquin JS, Carrillo JD, Twizeyimana M, Peacock BB, Sugino KY, Na F, Wang DH, Kabashima JN & Eskalen A (2018) Chemical management of invasive shot hole borer and Fusarium dieback in California sycamore (*Platanus racemosa*) in southern California. *Plant Disease* **102**: 1307-1315.

Mendel Z, Protasov A, Sharon M, Zveibil A, Yehuda SB, O'Donnell K, Rabaglia R, Wysoki M & Freeman S (2012) An Asian ambrosia beetle *Euwallacea fornicatus* and its novel symbiotic fungus *Fusarium* sp. pose a serious threat to the Israeli avocado industry. *Phytoparasitica* **40**: 235-238.

Muis A (2016) Biomass production and formulation of *Bacillus subtilis* for biological control. *Indonesian Journal of Agricultural Science* **7:** 51-55

Nahrung HF, Ramsden M, Hayes RA, Francis LP & Griffiths MW (2016) Performance of *Sirex noctilio*'s biocontrol agent *Deladenus siricidicola*, in known and predicted hosts. *Biological Control* **103**: 54-61.

Nene Y & Thapliyal PN (1993) *Fungicides in plant disease control*. International Science Publisher.

Niks RE, Parlevliet J, Lindhout P & Bai Y (2011) *Breeding crops with resistance to diseases and pests*. Wageningen Academic Publishers.

Ongena M & Jacques P (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends in Microbiology* **16**: 115-125.

Paap T, De Beer ZW, Migliorini D, Nel W & Wingfield MJ (2018) The polyphagous shot hole borer (PSHB) and its fungal symbiont *Fusarium euwallaceae*: a new invasion in South Africa. *Australasian Plant Pathology* **47**: 231-237.

Perrings C, Dehnen-Schmutz K, Touza J & Williamson M (2005) How to manage biological invasions under globalisation. *Trends in Ecology & Evolution* **20**: 212-215.

Ploetz RC, Hulcr J, Wingfield MJ & De Beer ZW (2013) Destructive tree diseases associated with ambrosia and bark beetles: black swan events in tree pathology? *Plant Disease* **97**: 856-872.

Sarwar M (2015) The killer chemicals as controller of agriculture insect pests: The conventional insecticides. *International Journal of Chemical and Biomolecular Science* **1**: 141-147.

Sharma B & Kumar P (2009) *In vitro* antifungal potency of some plant extracts against Fusarium oxysporum. *International Journal of Green Pharmacy (IJGP)* **3**.

Shea K, Thrall PH & Burdon JJ (2000) An integrated approach to management in epidemiology and pest control. *Ecology Letters* **3**: 150-158.

Sundh I & Goettel MS (2013) Regulating biocontrol agents: a historical perspective and a critical examination comparing microbial and macrobial agents. *Biological Control* **58**: 575-593.

van den Berg N, du Toit M, Morgan S, Fourie G & de Beer ZW (2019) First report of *Fusarium euwallaceae* causing necrotic lesions on *Persea americana* in South Africa. *Plant Disease* **10**: 18-1818-PDN.

SUPPLEMENTARY INFORMATION

Table 1: The three concentrations for each chemical agent used during the *in vitro* control assay, against *Fusarium euwallaceae*. The use of all these fungicides were based on the *in planta* EC50 described by Mayorquin *et al.* (2018). These EC50 value were used as the baseline concentrations, from which two higher concentrations followed, towards trying to achieve complete inhibition of *F. euwallaceae* mycelia.

Fungicide Common Name	**Concentration 1 (*in planta* EC50)** (µg/ml)	**Concentration 2** (µg/ml)	**Concentration 3** (µg/ml)
Fluazinam	0.05	0.10	0.15
Metconazole	25	40	60
Tebuconazole	100	150	200
Propiconazole	150	350	500

Table 2: Measurements of the lesion lengths, lesion length means, standard deviations and standard errors obtained from the control and the artificially inoculated Ettinger plantlets. As expected, the control plants had significantly smaller lesions, compared to all of the other groups. From the results, it is also clear that *F. euwallaceae* caused larger lesions than *G. euwallaceae,* but that a combination of the two fungi, in a single co-inoculation site, yielded lesions larger than both *F. euwallaceae* alone and *G. euwallaceae* alone.

Ettinger Cultivar (18 Months old - 1.5 m tall)				
Number #	Lesion lengths, infected with *F. euwallaceae* only (cm)	Lesion lengths, infected with G*. euwallaceae* only (cm)	Lesion lengths of co-inoculations (cm)	Lesion lengths of controls (cm)
Seedling 1	3,1	3	8	1,4
Seedling 2	6,8	6,2	4,3	1,3
Seedling 3	4,1	5,9	13	0,8
Seedling 4	6,2	2	7,8	
Seedling 5	5,5	4,8	5,5	
Seedling 6	6,3	6,8		
Seedling 7	10	5,4		
Overall average	**6**	**4,87**	**7,72**	**1,17**
Standard Deviation	2,20	1,76	3,34	0,32
Standard Error	0,83	0,67	1,49	0,19

Table 3: Measurements of the lesion lengths, lesion length means, standard deviations and standard errors obtained from the control and the artificially inoculated Edranol seedling plants. As expected, the control plants had significantly smaller lesions, compared to all of the other groups. From the results, it is also clear that *F. euwallaceae* caused larger lesions than *G. euwallaceae,* but that a combination of the two fungi, in a single co-inoculation site, yielded larger lesions equal to the sum of both the lesions caused by *F. euwallaceae* alone and *G. euwallaceae* alone.

Edranol Cultivar (8 Months old - 60 cm tall)				
Number #	Lesion lengths, infected with *F. euwallaceae* only (cm)	Lesion lengths, infected with G. *euwallaceae* only (cm)	Lesion lengths of co-inoculations (cm)	Lesion lengths of controls (cm)
Seedling 1	5,5	4,2	9	1,1
Seedling 2	2,9	2	5,5	2,1
Seedling 3	3,1	3,5	6,2	1
Seedling 4	4	1,9	5,9	
Seedling 5	2,5	2,3	5,7	
Seedling 6	5,2	1,8		
Seedling 7	2,6	2,4		
Overall average	**3,69**	**2,59**	**6,46**	**1,40**
Standard Deviation	1,24	0,91	1,44	0,61
Standard Error	0,47	0,34	0,65	0,35

Table 4: The statistical analysis of the data obtained from the measurements of the lesions from the Ettinger seedlings, including: the control seedlings, the *F. euwallaceae* only inoculations, the *G. euwallaceae* only inoculations and the co-inoculation of both fungi. The data was analysed using GraphPad Prism 8 and followed the conclusion of Gaussian (normal) distribution, by means of the Shapiro-Wilk test. Thereafter, an ordinary 1-way-ANOVA and Tukey's multiple comparisons test were conducted. This test allowed for the determination of significant difference between the mean of the different data sets and that of the mean for the controls.

Ettinger					
Shapiro-Wilk test	*Fusarium* only	*Graphium* only	Co-Inoculation	Control	
W	0,9421	0,912	0,9145	0,871	
P value	0,6575	0,41	0,4954	0,2983	
Passed normality test (alpha=0.05)?	**Yes**	**Yes**	**Yes**	**Yes**	
P value summary	ns	ns	ns	ns	
Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	**Significant?**	**Summary**	Adjusted P Value
Infected with Fusarium vs. Infected with Graphium	1,129	-2,294 to 4,552	**Yes**	*	0,7884
Infected with Fusarium vs. Co-Inoculation	-1,72	-5,470 to 2,030	**Yes**	*	0,5768
Infected with Fusarium vs. Control	4,833	0,4143 to 9,252	**Yes**	*	0,0293
Infected with Graphium vs. Co-Inoculation	-2,849	-6,598 to 0,9011	**Yes**	*	0,1762
Infected with Graphium vs. Control	3,705	-0,7143 to 8,124	**Yes**	*	0,1193
Co-Inoculation vs. Control	6,553	1,877 to 11,23	**Yes**	**	0,0046

Table 5: The statistical analysis of the data obtained from the measurements of the lesions from the Edranol seedlings, including: the control seedlings, the *F. euwallaceae* only inoculations, the *G. euwallaceae* only inoculations and the co-inoculation of both fungi. The data was analysed using GraphPad Prism 8 and followed the conclusion of Gaussian (normal) distribution, by means of the Shapiro-Wilk test. Thereafter, an ordinary 1-way-ANOVA and Tukey's multiple comparisons test were conducted. This test allowed for the determination of significant difference between the mean of the different data sets and that of the mean for the controls.

Edranol				
Shapiro-Wilk test	*Fusarium* only	*Graphium* only	Co-Inoculation	Control
W	0,8571	0,8316	0,7186	0,8176
P value	0,1425	0,0828	0,0148	0,1572
Passed normality test (alpha=0.05)?	**Yes**	**Yes**	**Yes**	**Yes**
P value summary	ns	ns	*	ns

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	**Significant?**	**Summary**	Adjusted P Value
Infected with Fusarium vs. Infected with Graphium	1,1	-0,6184 to 2,818	**Yes**	*	0,3013
Infected with Fusarium vs. Co-Inoculation	-2,774	-4,657 to -0,8919	Yes	**	0,003
Infected with Fusarium vs. Control	**2,286**	**0,06727 to 4,504**	**Yes**	*	**0,0421**
Infected with Graphium vs. Co-Inoculation	-3,874	-5,757 to -1,992	Yes	****	<0,0001
Infected with Graphium vs. Control	**1,186**	**-1,033 to 3,404**	**Yes**	*	**0,452**
Co-Inoculation vs. Control	**5,06**	**2,712 to 7,408**	**Yes**	****	**<0,0001**

Table 6: The measurements and mean measurements of *F. euwallaceae* mycelial growth, plated onto different concentrations of ½ PDA, amended with fluazinam, at 3 DAP. It is clear how the higher concentrations of fluazinam that were applied, showed more mycelial inhibition, even at 3 DAP. The measurements and the mean of the control plates are also indicated.

Fluazinam **3 DAP**	Measurements (mm) for Concentration 1 (0.05 µg/ml)	Mean Measurement (mm)	Measurements (mm) for Concentration 2 (0.10 µg/ml)	Mean Measurement (mm)	Measurements (mm) for Concentration 3 (0.15 µg/ml)	Mean Measurement (mm)
Sample 1	25 x 23	24,0	20 x 22	21	20 x 19	19,5
Sample 2	23 x 24	23,5	22 x 24	23	18 x 21	19,5
Sample 3	25 x 23	24,0	23 x 24	23,5	20 x 20	20
Sample 4	24 x 22	23,0	19 x 18	18,5	19 x 18	18,5
Sample 5	23 x 19	21,0	20 x 23	21,5	24 x 19	21,5
Sample 6	26 x 22	24,0	24 x 26	25	20 x 19	19,5
Sample 7	33 x 22	27,5	19 x 22	20,5	18 x 17	17,5
Sample 8	24 x 21	22,5	29 x 17	23	16 x 17	16,5
	Overall average for Concentration 1	23,69	**Overall average for Concentration 2**	22	**Overall average for Concentration 3**	19,06
Control 1	32 x 36	34				
Control 2	35 x 36	35,5				
	Overall average for controls	34,75				

Table 7: The measurements and mean measurements of *F. euwallaceae* mycelial growth, plated onto different concentrations of ½ PDA, amended with fluazinam, at 6 DAP. It is clear how the higher concentrations of fluazinam that were applied, showed more mycelial inhibition, at 6 DAP. The measurements and the mean of the control plates are also indicated.

Fluazinam **6 DAP**	Measurements (mm) for Concentration 1 (0.05 µg/ml)	Mean Measurement (mm)	Measurements (mm) for Concentration 2 (0.10 µg/ml)	Mean Measurement (mm)	Measurements (mm) for Concentration 3 (0.15 µg/ml)	Mean Measurement (mm)
Sample 1	37 x 36	36,5	32 x 35	33,5	30 x 31	30,5
Sample 2	40 x 36	38	36 x 35	35,5	28 x 31	29,5
Sample 3	38 x 36	37	38 x 40	39	35 x 30	32,5
Sample 4	35 x 34	34,5	40 x 29	34,5	28 x 29	28,5
Sample 5	36 x 31	33,5	34 x 38	36	35 x 34	34,5
Sample 6	34 x 36	35	42 x 37	39,5	30 x 30	30
Sample 7	35 x 37	36	34 x 34	34	26 x 29	27,5
Sample 8	40 x 44	42	42 x 35	38,5	29 x 27	28
	Overall average for Concentration 1	36,56	**Overall average for Concentration 2**	36,31	**Overall average for Concentration 3**	30,13
Control 1	58 x 57	57,5				
Control 2	59 x 56	57,5				
	Overall average for controls	57,5				

Table 8: The statistical analysis of the data obtained from mean measurements of *F. euwallaceae* mycelia, plated onto different concentrations of ½ PDA, amended with fluazinam, at 3 DAP. The data was analysed using GraphPad Prism 8 and followed the conclusion of Gaussian (normal) distribution, by means of the Shapiro-Wilk test. Thereafter, an ordinary 1-way-ANOVA and Tukey's multiple comparisons test were conducted. This test allowed for the determination of significant difference between the mean of the replicates and that of the mean for the controls.

Fluazinam 3 DAP	Concentration 1 (0.05 µg/ml)	Concentration 2 (0.10 µg/ml)	Concentration 3 (0.15 µg/ml)	**Control**
Standard Deviation	1,850434312	2,035400978	1,545442609	1,060660172
Standard Error	0,654227325	0,719622917	0,546396474	0,75

Shapiro-Wilk test	Concentration 1 (0.05 µg/ml)	Concentration 2 (0.10 µg/ml)	Concentration 3 (0.15 µg/ml)	**Control**
W	0,8869	0,9748	0,9528	N too small
P value	0,2191	0,9325	0,7393	
Passed normality test (alpha=0.05)?	**Yes**	**Yes**	**Yes**	

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	**Significant?**	**Summary**	Adjusted P Value
Concentration 1 (0.05 µg/ml) vs. Concentration 2 (0.10 µg/ml)	1,688	-0,8034 to 4,178	No	ns	0,2646
Concentration 1 (0.05 µg/ml) vs. Concentration 3 (0.15 µg/ml)	4,625	2,134 to 7,116	Yes	***	0,0002
Concentration 1 (0.05 µg/ml) vs. Control	**-11,06**	**-15,00 to -7,124**	**Yes**	****	**<0,0001**
Concentration 2 (0.10 µg/ml) vs. Concentration 3 (0.15 µg/ml)	2,938	0,4466 to 5,428	Yes	*	0,0169
Concentration 2 (0.10 µg/ml) vs. Control	**-12,75**	**-16,69 to -8,811**	**Yes**	****	**<0,0001**
Concentration 3 (0.15 µg/ml) vs. Control	**-15,69**	**-19,63 to -11,75**	**Yes**	****	**<0,0001**

Table 9: The statistical analysis of the data obtained from mean measurements of *F. euwallaceae* mycelia, plated onto different concentrations of ½ PDA, amended with fluazinam, at 6 DAP. The data was analysed using GraphPad Prism 8 and followed the conclusion of Gaussian (normal) distribution, by means of the Shapiro-Wilk test. Thereafter, an ordinary 1-way-ANOVA and Tukey's multiple comparisons test were conducted. This test allowed for the determination of significant difference between the mean of the replicates and that of the mean for the controls.

Fluazinam 6 DAP	Concentration 1 (0.05 µg/ml)	Concentration 2 (0.10 µg/ml)	Concentration 3 (0.15 µg/ml)	**Control**
Standard Deviation	2,624574795	2,374530029	2,371708245	0
Standard Error	0,927927318	0,839523143	0,838525492	0

Shapiro-Wilk test	Concentration 1 (0.05 µg/ml)	Concentration 2 (0.10 µg/ml)	Concentration 3 (0.15 µg/ml)	**Control**
W	0,9112	0,8941	0,9257	N too small
P value	0,3627	0,2555	0,4776	
Passed normality test (alpha=0.05)?	**Yes**	**Yes**	**Yes**	
P value summary	ns	ns	ns	

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	**Significant?**	**Summary**	Adjusted P Value
Concentration 1 (0.05 µg/ml) vs. Concentration 2 (0.10 µg/ml)	0,25	-3,087 to 3,587	No	ns	0,9967
Concentration 1 (0.05 µg/ml) vs. Concentration 3 (0.15 µg/ml)	6,438	3,101 to 9,774	Yes	***	0,0001
Concentration 1 (0.05 µg/ml) vs. Control	**-20,94**	**-26,21 to -15,66**	**Yes**	****	**<0,0001**
Concentration 2 (0.10 µg/ml) vs. Concentration 3 (0.15 µg/ml)	6,188	2,851 to 9,524	Yes	***	0,0002
Concentration 2 (0.10 µg/ml) vs. Control	**-21,19**	**-26,46 to -15,91**	**Yes**	****	**<0,0001**
Concentration 3 (0.15 µg/ml) vs. Control	**-27,38**	**-32,65 to -22,10**	**Yes**	****	**<0,0001**

Table 10: The measurements and mean measurements of *F. euwallaceae* mycelial growth, plated onto different concentrations of ½ PDA, amended with propiconazole, at 3 DAP. It is clear how the higher concentrations of propiconazole that were applied, showed more mycelial inhibition, even at 3 DAP. The area marked in pink indicate an absence of measurements for 500 µg/ml, as complete mycelial inhibition was observed. The measurements and the mean of the control plates are also indicated.

Propiconazole **3 DAP**	Measurements (mm) for Concentration 1 (150 µg/ml)	Mean Measurement (mm)	Measurements (mm) for Concentration 2 (350 µg/ml)	Mean Measurement (mm)	Measurements (mm) for Concentration 3 (500 µg/ml)	Mean Measurement (mm)
Sample 1	15 x 15	15	11 x 11	11		
Sample 2	13 x 14	13,5	10 x 11	10,5		
Sample 3	16 x 17	16,5	16 x 12	14		
Sample 4	16 x 14	15	11 x 12	11,5		
Sample 5	15 x 16	15,5	10 x 10	10		
Sample 6	16 x 15	15,5	10 x 11	10,5		
Sample 7	14 x 11	12,5	11 x 11	11		
Sample 8	16 x 14	15	10 x 11	10,5		
	Overall average for Concentration 1	14,81	**Overall average for Concentration 2**	11,13		
Control 1	36 x 35	35,5				
Control 2	36 x 33	34,5				
	Overall average for controls	35				

Table 11: The measurements and mean measurements of *F. euwallaceae* mycelial growth, plated onto different concentrations of ½ PDA, amended with propiconazole, at 6 DAP. It is clear how the higher concentrations of propiconazole that were applied, showed more mycelial inhibition, even at 6 DAP. The concentration at which complete mycelial inhibition was previously observed, at 3 DAP (500 µg/ml), started to indicate the presence of actively growing *F. euwallaceae* mycelia, at 6 DAP. The measurements and the mean of the control plates are also indicated.

Propiconazole **6 DAP**	Measurements (mm) for Concentration 1 (150 µg/ml)	Mean Measurement (mm)	Measurements (mm) for Concentration 2 (350 µg/ml)	Mean Measurement (mm)	Measurements (mm) for Concentration 3 (500 µg/ml)	Mean Measurement (mm)
Sample 1	25 x 25	25	15 x 15	15	13 x 12	12,5
Sample 2	19 x 20	19,5	15 x 15	15	13 x 11	12
Sample 3	23 x 22	22,5	16 x 20	18	13 x 13	13
Sample 4	23 x 23	23	12 x 13	12,5	10 x 12	11
Sample 5	25 x 25	25	13 x 15	14	13 x 13	13
Sample 6	25 x 22	23,5	15 x 16	15,5	12 x 10	11
Sample 7	27 x 26	26,5	17 x 15	16	11 x 12	11,5
Sample 8	26 x 26	26	16 x 16	16	10 x 11	10,5
	Overall average for Concentration 1	23,88	**Overall average for Concentration 2**	15,25	**Overall average for Concentration 3**	11,81
Control 1	60 x 61	60,5				
Control 2	60 x 60	60				
	Overall average for controls	60,25				

Table 12: The statistical analysis of the data obtained from mean measurements of *F. euwallaceae* mycelia, plated onto different concentrations of ½ PDA, amended with propiconazole, at 3 DAP. The data was analysed using GraphPad Prism 8 and followed the conclusion of Gaussian (normal) distribution, by means of the Shapiro-Wilk test. Thereafter, an ordinary 1-way-ANOVA and Tukey's multiple comparisons test were conducted. This test allowed for the determination of significant difference between the mean of the replicates and that of the mean for the controls.

Propiconazole 3 DAP	Concentration 1 (150 µg/ml)	Concentration 2 (350 µg/ml)	Concentration 3 (500 µg/ml)	**Control**
Standard Deviation	1,251784441	1,246423455	0	0,70710678
Standard Error	0,442572633	0,440677239	0	0,5

Shapiro-Wilk test	Concentration 1 (150 µg/ml)	Concentration 2 (350 µg/ml)	Concentration 3 (500 µg/ml)	**Control**
W	0,8991	0,7481	Invalid input	N too small
P value	0,2837	0,0078		
Passed normality test (alpha=0.05)?	**Yes**	**Yes**		
P value summary	ns	**		

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	**Significant?**	**Summary**	Adjusted P Value
Concentration 1 (150 µg/ml) vs. Concentration 2 (350 µg/ml)	3,688	2,288 to 5,087	Yes	****	<0,0001
Concentration 1 (150 µg/ml) vs. Concentration 3 (500 µg/ml)	14,81	13,41 to 16,21	Yes	****	<0,0001
Concentration 1 (150 µg/ml) vs. Control	**-20,19**	**-22,40 to -17,98**	**Yes**	****	**<0,0001**
Concentration 2 (350 µg/ml) vs. Concentration 3 (500 µg/ml)	11,13	9,726 to 12,52	Yes	****	<0,0001
Concentration 2 (350 µg/ml) vs. Control	**-23,88**	**-26,09 to -21,66**	**Yes**	****	**<0,0001**
Concentration 3 (500 µg/ml) vs. Control	**-35**	**-37,21 to -32,79**	**Yes**	****	**<0,0001**

Table 13: The statistical analysis of the data obtained from mean measurements of *F. euwallaceae* mycelia, plated onto different concentrations of ½ PDA, amended with propiconazole, at 6 DAP. The data was analysed using GraphPad Prism 8 and followed the conclusion of Gaussian (normal) distribution, by means of the Shapiro-Wilk test. Thereafter, an ordinary 1-way-ANOVA and Tukey's multiple comparisons test were conducted. This test allowed for the determination of significant difference between the mean of the replicates and that of the mean for the controls.

Propiconazole 6 DAP	Concentration 1 (150 µg/ml)	Concentration 2 (350 µg/ml)	Concentration 3 (500 µg/ml)	**Control**
Standard Deviation	2,263846285	1,603567451	0,961304917	0,35355339
Standard Error	0,80039053	0,56694671	0,339872613	0,25

Shapiro-Wilk test	Concentration 1 (150 µg/ml)	Concentration 2 (350 µg/ml)	Concentration 3 (500 µg/ml)	**Control**
W	0,9304	0,9632	0,9175	N too small
P value	0,5199	0,8398	0,4097	
Passed normality test (alpha=0.05)?	**Yes**	**Yes**	**Yes**	
P value summary	ns	ns	ns	

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	**Significant?**	**Summary**	Adjusted P Value
Concentration 1 (150 µg/ml) vs. Concentra	8,625	6,323 to 10,93	Yes	****	<0,0001
Concentration 1 (150 µg/ml) vs. Concentra	12,06	9,761 to 14,36	Yes	****	<0,0001
Concentration 1 (150 µg/ml) vs. Control	**-36,38**	**-40,01 to -32,74**	**Yes**	****	**<0,0001**
Concentration 2 (350 µg/ml) vs. Concentra	3,438	1,136 to 5,739	Yes	**	0,0022
Concentration 2 (350 µg/ml) vs. Control	**-45**	**-48,64 to -41,36**	**Yes**	****	**<0,0001**
Concentration 3 (500 µg/ml) vs. Control	**-48,44**	**-52,08 to -44,80**	**Yes**	****	**<0,0001**

Table 14: The measurements and mean measurements of *F. euwallaceae* mycelial growth, dual-cultured onto ½ PDA, with B-Rus, *Bacillus* spp. culture powder, at 3 DAP. The measurements and the mean of the control plates are also indicated. As expected, the average for the mycelial growth on the control plates was higher than the average for the mycelial growth on the dual culture plates.

***Bacillus spp.* (B-Rus) 3 DAP**	Measurements (mm)	Mean Measurement (mm)
Sample 1	21 x 23	22
Sample 2	19 x 24	21,5
Sample 3	17 x 19	18
Sample 4	18 x 20	19
Sample 5	18 x 24	21
Sample 6	20 x 25	22,5
Sample 7	11 x 12	11,5
Sample 8	21 x 17	19
Sample 9	18 x 18	18
Sample 10	22 x 19	20,5
Overall average for *Fusarium*		19,3
Control 1	47 x 39	43
Control 2	37 x 42	39,5
Control 3	36 x 37	36,5
Overall average for controls		39,67

Table 15: The measurements and mean measurements of *F. euwallaceae* mycelial growth, dual-cultured onto ½ PDA, with B-Rus, *Bacillus* spp. culture powder, at 6 DAP. The measurements and the mean of the control plates are also indicated. As expected, the average for the mycelial growth on the control plates was higher than the average for the mycelial growth on the dual culture plates. The mycelial growth diameter on the dual culture plates remained unchanged at 6 DAP.

***Bacillus spp.* (B-Rus) 6 DAP**	Measurements (mm)	Mean Measurement (mm)
Sample 1	21 x 23	22
Sample 2	19 x 24	21,5
Sample 3	17 x 19	18
Sample 4	18 x 20	19
Sample 5	18 x 24	21
Sample 6	20 x 25	22,5
Sample 7	11 x 12	11,5
Sample 8	21 x 17	19
Sample 9	18 x 18	18
Sample 10	22 x 19	20,5
Overall average for *Fusarium*		19,3
Control 1	65 x 70	67,5
Control 2	64 x 63	63,5
Control 3	67 x 68	67,5
Overall average for controls		66,17

Table 16: The statistical analysis of the data obtained from mean measurements of *F. euwallaceae* mycelia, dual-cultured onto ½ PDA, with B-Rus, *Bacillus* spp. culture powder, at 3 DAP. The data was analysed using GraphPad Prism 8 and followed the conclusion of Gaussian (normal) distribution, by means of the Shapiro-Wilk test. Thereafter, an ordinary 1-way-ANOVA and Tukey's multiple comparisons test were conducted. This test allowed for the determination of significant difference between the mean of the replicates and that of the mean for the controls.

Bacillus **(B-Rus) 3 DAP**	**Mycelial growth on dual plate**	**Controls**
Standard Deviation	3,181544	3,253203549
Standard Error	1,006092552	1,878237945

Shapiro-Wilk test	**Mycelial growth on dual plate**	**Controls**
W	0,8275	0,998
P value	0,0313	0,9152
Passed normality test (alpha=0.05)?	**Yes**	**Yes**
P value summary	*	ns

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	**Significant?**	**Summary**	Adjusted P Value
Mycelial growth on dual plate vs. Controls	**-20,37**	**-24,31 to -16,42**	**Yes**	********	**<0,0001**

Table 17: The statistical analysis of the data obtained from mean measurements of *F. euwallaceae* mycelia, dual-cultured onto ½ PDA, with B-Rus, *Bacillus* spp. culture powder, at 6 DAP. The data was analysed using GraphPad Prism 8 and followed the conclusion of non-Gaussian distribution, by means of the Shapiro-Wilk test. Thereafter, a non-parametric Kraskal-Willis test and Dunn's multiple comparisons test were conducted. This test allowed for the determination of significant difference between the mean of the replicates and that of the mean for the controls.

Bacillus **(B-Rus) 6 DAP**	**Mycelial growth on dual plate**	**Controls**
Standard Deviation	3,181544	2,3094
Standard Error	1,006092552	1,33333

Shapiro-Wilk test	**Mycelial growth on dual plate**	**Controls**
W	0,8275	0,75
P value	0,0313	
Passed normality test (alpha=0.05)?	**No**	
P value summary	*	

Dunn's multiple comparisons test	Mean Diff,	95,00% CI of diff,	**Significant?**	**Summary**	Adjusted P Value
Mycelial growth on dual plate vs. Controls	**-46,87**	**-50,62 to -43,11**	**Yes**	****	**<0,0001**

Table 18: The measurements and mean measurements of *F. euwallaceae* mycelial growth, dual-cultured onto ½ PDA, with Mity-Gro, *Bacillus* spp. culture powder, at 3 DAP. The measurements and the mean of the control plates are also indicated. As expected, the average for the mycelial growth on the control plates was higher than the average for the mycelial growth on the dual culture plates.

***Bacillus spp.* (Mity Gro) 3 DAP**	Measurements (mm)	Mean Measurement (mm)
Sample 1	32 x 32	32
Sample 2	29 x 30	29,5
Sample 3	28 x 20	24
Sample 4	30 x 35	32,5
Sample 5	31 x 30	30,5
Sample 6	43 x 30	36,5
Sample 7	30 x 26	28
Sample 8	30 x 34	32
Sample 9	29 x 31	30
Sample 10	35 x 35	35
Overall average for *Fusarium*		31
Control 1	44 x 38	41
Control 2	39 x 42	40,5
Control 3	38 x 32	35
Overall average for controls		38,83

Table 19: The measurements and mean measurements of *F. euwallaceae* mycelial growth, dual-cultured onto ½ PDA, with Mity-Gro, *Bacillus* spp. culture powder, at 6 DAP. The measurements and the mean of the control plates are also indicated. As expected, the average for the mycelial growth on the control plates was higher than the average for the mycelial growth on the dual culture plates. The mycelial growth diameter on the dual culture plates remained unchanged at 6 DAP.

Bacillus spp. (Mity Gro) **6 DAP**	Measurements (mm)	Mean Measurement (mm)
Sample 1	32 x 32	32
Sample 2	29 x 30	29,5
Sample 3	28 x 20	24
Sample 4	30 x 35	32,5
Sample 5	31 x 30	30,5
Sample 6	43 x 30	36,5
Sample 7	30 x 26	28
Sample 8	30 x 34	32
Sample 9	29 x 31	30
Sample 10	35 x 35	35
Overall average for *Fusarium*		31
Control 1	68 x 70	69
Control 2	65 x 65	65
Control 3	64 x 70	67
Overall average for controls		67

Table 20: The statistical analysis of the data obtained from mean measurements of *F. euwallaceae* mycelia, dual-cultured onto ½ PDA, with Mity-Gro, *Bacillus* spp. culture powder, at 3 DAP. The data was analysed using GraphPad Prism 8 and followed the conclusion of Gaussian (normal) distribution, by means of the Shapiro-Wilk test. Thereafter, an ordinary 1-way-ANOVA and Tukey's multiple comparisons test were conducted. This test allowed for the determination of significant difference between the mean of the replicates and that of the mean for the controls.

Bacillus **(Mity Gro) 3 DAP**	**Mycelial growth on dual plate**	**Controls**
Standard Deviation	3,527668415	3,329164059
Standard Error	1,115546702	1,922093766

Shapiro-Wilk test	**Mycelial growth on dual plate**	**Controls**
W	0,97	0,812
P value	0,8907	0,1436
Passed normality test (alpha=0.05)?	**Yes**	**Yes**
P value summary	ns	ns

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	**Significant?**	**Summary**	Adjusted P Value
Mycelial growth on dual plate vs. Controls	**-7,833**	**-12,15 to -3,520**	**Yes**	***	**0,0005**

Table 21: The statistical analysis of the data obtained from mean measurements of *F. euwallaceae* mycelia, dual-cultured onto ½ PDA, with Mity-Gro, *Bacillus* spp. culture powder, at 6 DAP. The data was analysed using GraphPad Prism 8 and followed the conclusion of Gaussian (normal) distribution, by means of the Shapiro-Wilk test. Thereafter, an ordinary 1-way-ANOVA and Tukey's multiple comparisons test were conducted. This test allowed for the determination of significant difference between the mean of the replicates and that of the mean for the controls.

Bacillus **(Mity Gro) 6 DAP**	**Mycelial growth on dual plate**	**Controls**
Standard Deviation	3,527668415	2
Standard Error	1,115546702	1,154700538

Shapiro-Wilk test	**Mycelial growth on dual plate**	**Controls**
W	0,97	1
P value	0,8907	>0,9999
Passed normality test (alpha=0.05)?	**Yes**	**Yes**
P value summary	ns	ns

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	**Significant?**	**Summary**	Adjusted P Value
Mycelial growth on dual plate vs. Controls	**-36**	**-40,08 to -31,92**	**Yes**	********	**<0,0001**

Table 22: The measurements and mean measurements of *F. euwallaceae* mycelial growth, dual-cultured onto ½ PDA, with *Trichoderma* spp. culture powder, at 3 DAP. The measurements and the mean of the control plates are also indicated. As expected, the average for the mycelial growth on the control plates was higher than the average for the mycelial growth on the dual culture plates.

Trichoderma spp. **3 DAP**	Measurement (mm)
Sample 1	13
Sample 2	14
Sample 3	10
Sample 4	12
Sample 5	12
Sample 6	9
Sample 7	11
Sample 8	13
Sample 9	13
Sample 10	15
Overall average for *Fusarium*	12,2
Control 1	19
Control 2	16
Control 3	18
Overall average for controls	17,67

Table 23: The measurements and mean measurements of *F. euwallaceae* mycelial growth, dual-cultured onto ½ PDA, with *Trichoderma* spp. culture powder, at 3 DAP. The measurements and the mean of the control plates are also indicated. As expected, the average for the mycelial growth on the control plates was higher than the average for the mycelial growth on the dual culture plates. The mycelial *F. euwallaceae* growth diameter on the dual culture plates remained unchanged at 6 DAP.

Trichoderma spp. **6 DAP**	Measurement (mm)
Sample 1	13
Sample 2	14
Sample 3	10
Sample 4	12
Sample 5	12
Sample 6	9
Sample 7	11
Sample 8	13
Sample 9	13
Sample 10	15
Overall average for *Fusarium*	12,2
Control 1	31
Control 2	28
Control 3	32
Overall average for controls	30,33

Table 24: The statistical analysis of the data obtained from mean measurements of *F. euwallaceae* mycelia, dual-cultured onto ½ PDA, with *Trichoderma* spp. culture powder, at 3 DAP. The data was analysed using GraphPad Prism 8 and followed the conclusion of Gaussian (normal) distribution, by means of the Shapiro-Wilk test. Thereafter, an ordinary 1-way-ANOVA and Tukey's multiple comparisons test were conducted. This test allowed for the determination of significant difference between the mean of the replicates and that of the mean for the controls.

***Trichoderma* 3 DAP**	***Fusarium* Mycelial growth on dual plate**	**Controls**
Standard Deviation	1,813529401	1,527525232
Standard Error	0,573488351	0,881917104

Shapiro-Wilk test	***Fusarium* Mycelial growth on dual plates**	**Controls**
W	0,9669	0,9231
P value	0,8606	0,4633
Passed normality test (alpha=0.05)?	**Yes**	**Yes**
P value summary	ns	ns

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	**Significant?**	**Summary**	Adjusted P Value
***Fusarium* Mycelial growth on dual plate vs. Controls**	**-5,778**	**-7,805 to -3,750**	**Yes**	********	**<0,0001**

Table 25: The statistical analysis of the data obtained from mean measurements of *F. euwallaceae* mycelia, dual-cultured onto ½ PDA, with *Trichoderma* spp. culture powder, at 6 DAP. The data was analysed using GraphPad Prism 8 and followed the conclusion of Gaussian (normal) distribution, by means of the Shapiro-Wilk test. Thereafter, an ordinary 1-way-ANOVA and Tukey's multiple comparisons test were conducted. This test allowed for the determination of significant difference between the mean of the replicates and that of the mean for the controls.

***Trichoderma* 6 DAP**	***Fusarium* Mycelial growth on dual plates**	**Controls**
Standard Deviation	1,813529401	2,081665999
Standard Error	0,573488351	1,201850425

Shapiro-Wilk test	***Fusarium* Mycelial growth on dual plates**	**Controls**
W	0,9669	0,9231
P value	0,8606	0,4633
Passed normality test (alpha=0.05)?	**Yes**	**Yes**
P value summary	ns	ns

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	**Significant?**	**Summary**	Adjusted P Value
***Fusarium* Mycelial growth on dual plate vs. Controls**	**-18,13**	**-20,44 to -15,83**	**Yes**	********	**<0,0001**

Chapter 4:

General Discussion and Recommendations for Future Work

The polyphagous shot hole borer – PSHB (*Euwallacea fornicatus)* is part of a group of insects known as ambrosia beetles, which has been shown to display persistent symbiotic relationships with more than one fungal organisms (Lynch *et al.*, 2016).This ambrosia beetle and its accompanying fungal organisms form a pest-complex that has been shown to cause Fusarium dieback (FD), on a diverse assortment of over 350 plant species, from over 50 families (Eskalen *et al.*, 2013). This pest complex has been shown to threaten forests, as well as ornamental and agricultural plant varieties, in its non-native distributions (Umeda *et al.*, 2016). One agriculturally-important plant species that has been shown to be seriously impacted, is the avocado fruit crop (*Persea americana)* in the United States of America and Israel (Mendel *et al.*, 2012, Freeman *et al.*, 2013). Globalisation and increased human movement have been shown to facilitate the spread of such economically-significant, insect pests, into areas where they have not previously occurred, thereby inferring threats to many local communities (Crowl *et al.*, 2008).

In a recent study, conducted in South Africa, aimed at detecting such emerging pests, the PSHB and one of its symbiotic fungal organisms (*Fusarium euwallaceae)* were detected causing damage to a variety of plants (Paap *et al.*, 2018). This early detection of this pest complex, in South Africa, meant that surveying for the presence of the PSHB, particularly in commercial avocado orchards, became an immediate priority. Along with monitoring for the spread of this pest, a lack of local knowledge meant that basic studies, on the biology of the causal agent/agents, were equally necessary. This was done towards considering ways to improve understanding of the plant-pathogen interactions of the various symbiotic fungi of the PSHB and to better implement management and control strategies, to limit the spread of the pest complex.

As part of continuous monitoring, this study successfully detected the PSHB and an accompanying fungal organism causing damage to an avocado tree, in Sandton, Johannesburg. The associated fungal organism (*F. euwallaceae)* was identified based on morphologic selection, as well as molecular analysis of the β-tubulin (*β-t*), and RNA polymerase II second largest subunit (*RBP2*) and translation elongation factor (*TEF 1-α*) gene regions and further phylogenetic inferences of the *TEF 1-α* gene sequences. Koch's postulates confirmed the pathogenicity of this fungal isolate to *P. americana*. Thereby providing a useful pipeline for the isolation, identification and pathogenicity determination of unknown fungal isolates. This finding is considered the first report of the PSHB and its fungal symbiont causing FD, to avocado, in South Africa (van den Berg *et al.*, 2019). This report was conducted and published to provide scientifically accurate awareness about the presence of this internationally invasive pest and to allude to the potential of damage that could occur, following the establishment of such a vector-borne pest complex.

Albeit that this study has yet to find evidence substantiating the presence of the PSHB in commercial avocado orchards, an inevitable arrival thereof is certainly possible. Difficulties regarding reliable extraction protocols and subsequent failure to amplify the *COX1* barcode gene, in this study, placed heavy and undesirable reliance on morphological identification of unknown beetles, meaning that the arrival of the PSHB, in commercial orchards, may already have occurred, but has yet to be detected. The lack of molecular approaches to identify these beetles may have resulted in a PSHB specimen being misidentified as a un-invasive, closely related organism. Therefore, there is a need for the development of a tailored DNA extraction protocol, in the future, that simultaneously yields high-quality DNA and is sensitive enough to extract DNA,

from a small amount of insect tissue, without the permanent loss of the entire beetle specimen.

Despite the lack of a reliable beetle DNA extraction method, during the surveying and monitoring process, this study found several other wood-boring beetles that were associated with damage to commercially active avocado trees. This study allowed for the detection of several wood-boring beetles, including some other ambrosia beetles from the Xyleborini tribe. Along with these beetles, several accompanying fungal organisms, many from the *Fusarium* genus, were also isolated from the associated damage to avocado, particularly stressed avocado trees. One particular beetle species, namely *Xylosandrus crassiusculus* was numerously detected and appeared to show a consistent association with *F. solani* spp. This beetle and the accompanying *F. solani* isolates were persistently associated with damage to stressed avocado trees and necessitates future research to determine the true species identity of the *F. solani* isolates and their potential pathogenicity to healthy avocado trees. Ambrosia beetles have not previously been considered a major threat to the avocado growing community, in South Africa, however, evidence from this study suggests the emergence of a potential threat. Due to increased biotic and abiotic stresses, as a result of climate change and human interference, many commercially active avocado trees may be placed under stress, thereby becoming conducive to the advent of such new threats (Avila-Quezada *et al.*, 2018).

The discovery of potential symbioses between non-PSHB beetles and pathogens other than *F. euwallaceae,* in this study, means that future metagenomic studies should be carried out to detect the profile of diversity from beetles and their associated

damage, on avocado. As opposed to artificial cultivation of fungal cultures, from primary isolations, onto nutrient media, metagenomics allows for the obtaining of all the genetic material, directly from environmental sampling, thereby revealing the actual microbial biodiversity that may have been missed by cultivation-based methods (Roossinck, 2015). This may allow for improved molecular profiling of the species diversity of the micro-organisms, associated with ambrosia beetles and their accompanying fungal symbionts (Cuadros-Orellana *et al.*, 2013). The application of metagenomic tools, in this way, may also allow for the characterisation of previously undescribed pathogenic species, that have potential mutualistic symbioses with one or more ambrosia beetles (Brady *et al.*, 2009).

One aspect that has remained understudied in literature, is the nature of the interactions between all of the fungal organisms consistently associated with the PSHB and their combined effect on host plants (Freeman *et al.*, 2016, Na *et al.*, 2018). Results from this study suggest that two of these symbiotic partners (*F. euwallaceae* and *Graphium euwallaceae*) are individually pathogenic and are individually able to initiate disease, but that the combined action of these organisms results in more severe disease symptoms. This is noteworthy as it represents a real-world scenario (Grosman *et al.*, 2019) and highlights both the lack of scientific studies, with regards to this pest complex, as well as the general complexity of the disease.

Besides considering the shared and individual *in planta* pathogenicity of the symbiotic partners of the PSHB, this study also briefly led to the consideration of various chemical and biological agents to control or treat instances of FD, specifically to the causal agent of the disease (*F. euwallaceae*). These studies showed promising

results, *in vitro*, in that each agent that was tested proved to significantly inhibit the growth of the fungal mycelia, even when applied individually. However, many practical uncertainties regarding these agents remain and must be further investigated, such as the effective mode of application, the long-term impact of the use on the environment and feasibility of application to different plant hosts. This emphasises the necessity of considering the results generated by this study with accompanying *in planta* observations. Results from this study can be compared with the *in planta* studies, done by Mayorquin *et al.* (2018). The *in vitro* results proved useful to identify candidate agents that could be considered for the treatment of FD, but also simultaneously concluded that results generated from *in vitro* studies may drastically underestimate the effective amount needed to achieve the same result, in field conditions.

This study also highlighted some shortcomings of the current and locally considered approaches to detecting and studying this pest complex. Many of the aspects mentioned within this thesis illustrate the value of currently available molecular techniques, for detection, diagnosis and identification. However, this study proved that even though molecular technologies are invaluable tools towards achieving accurate and unambiguous descriptions, molecular approaches cannot yet be used as a total replacement for morphologic studies. This being especially true for emerging threats that have yet to be sufficiently described in their native distributions and as a result, morphological considerations are instrumental for initial analysis and can often be used as a starting point to develop more sensitive and exact downstream molecular approaches (Summerell *et al.*, 2003).

Apparent losses, such as the direct impact on the economy, from damage to agricultural plant varieties, such as avocado, represents only a portion of the threat from the PSHB disease complex (Ploetz *et al.*, 2013). The PSHB and its fungal organisms and other ambrosia beetles have raised concerns as to the potential long-term impact on native and indigenous tree species. In a country, like South Africa, where plant-based biodiversity is abundant, the impact of a pest complex that can ultimately warp the natural structure of the plant-life within an ecosystem, must be crucially considered (Ploetz *et al.*, 2013). Invasions of this nature may result in the widespread loss of biodiversity and the natural succession of such an affected area may allow for the undesirable growth of more tolerant/resistant alien plant species. These unapparent and sometimes unnoticeable losses to biodiversity must be limited, particularly in an era where natural biodiversity is under threat from a variety of other factors.

The recent detection of the PSHB, on avocado and other hosts, in South Africa, the evidence of complexity exhibited by the disease lifestyle as a whole and the spread of the pest complex, means that a permanent monitoring process should be established to monitor the further spread and progression of this disease. The introduction of the PSHB into South Africa motivates the continuous risk posed by ineffective or inadequate national and international border biosecurity. To prevent the nationwide establishment and unforetold possible damage, integrated management strategies must be developed and long-term control approaches need to be sustainably undertaken to reduce the risk of further spread of this beetle-fungus, to the rest of the country or bordering countries and the greater African continent.

REFERENCES

Avila-Quezada GD, Esquivel JF, Silva-Rojas HV, Leyva-Mir SG, de Jesus Garcia-Avila C, Noriega-Orozco L, Rivas-Valencia P, Ojeda-Barrios D & Melgoza-Castillo A (2018) Emerging plant diseases under a changing climate scenario: Threats to our global food supply. *Emirates Journal of Food and Agriculture* **30:** 443-450.

Brady SF, Simmons L, Kim JH & Schmidt EW (2009) Metagenomic approaches to natural products from free-living and symbiotic organisms. *Natural Product Reports* **26**: 1488-1503.

Crowl TA, Crist TO, Parmenter RR, Belovsky G & Lugo AE (2008) The spread of invasive species and infectious disease as drivers of ecosystem change. *Frontiers in Ecology and the Environment* **6**: 238-246.

Cuadros-Orellana S, Leite LR, Smith A, Medeiros JD, Badotti F, Fonseca PL, Vaz AB, Oliveira G & Góes-Neto A (2013) Assessment of fungal diversity in the environment using metagenomics: a decade in review. *Fungal Genomics & Biology* **3**: 1-12.

Eskalen A, Stouthamer R, Lynch SC, Rugman-Jones PF, Twizeyimana M, Gonzalez A & Thibault T (2013) Host range of Fusarium dieback and its ambrosia beetle (Coleoptera: Scolytinae) vector in southern California. *Plant Disease* **97**: 938-951.

Freeman S, Sharon M, Maymon M, Mendel Z, Protasov A, Aoki T, Eskalen A & O'Donnell K (2013) *Fusarium euwallaceae* sp. nov.—a symbiotic fungus of

Euwallacea sp., an invasive ambrosia beetle in Israel and California. *Mycologia* **105**: 1595-1606.

Freeman S, Sharon M, Dori-Bachash M, Maymon M, Belausov E, Maoz Y, Margalit O, Protasov A & Mendel Z (2016) Symbiotic association of three fungal species throughout the life cycle of the ambrosia beetle *Euwallacea* nr. *fornicatus*. *Symbiosis* **68**: 115-128.

Grosman DM, Eskalen A & Brownie C (2019) Evaluation of Emamectin Benzoate and Propiconazole for Management of a New Invasive Shot Hole Borer (*Euwallacea* nr. *fornicatus*, Coleoptera: Curculionidae) and Symbiotic Fungi in California Sycamores. *Journal of Economic Entomology* **112:** 1267-1273.

Lynch SC, Twizeyimana M, Mayorquin JS, Wang DH, Na F, Kayim M, Kasson MT, Thu PQ, Bateman C & Rugman-Jones P (2016) Identification, pathogenicity and abundance of *Paracremonium pembeum* sp. nov. and *Graphium euwallaceae* sp. nov.—two newly discovered mycangial associates of the polyphagous shot hole borer (Euwallacea sp.) in California. *Mycologia* **108**: 313-329.

Mayorquin JS, Carrillo JD, Twizeyimana M, Peacock BB, Sugino KY, Na F, Wang DH, Kabashima JN & Eskalen A (2018) Chemical management of invasive shot hole borer and Fusarium dieback in California sycamore (*Platanus racemosa*) in southern California. *Plant Disease* **102**: 1307-1315.

Mendel Z, Protasov A, Sharon M, Zveibil A, Yehuda SB, O'Donnell K, Rabaglia R, Wysoki M & Freeman S (2012) An Asian ambrosia beetle *Euwallacea fornicatus* and its novel symbiotic fungus *Fusarium* sp. pose a serious threat to the Israeli avocado industry. *Phytoparasitica* **40**: 235-238.

Na F, Carrillo JD, Mayorquin JS, Ndinga-Muniania C, Stajich JE, Stouthamer R, Huang Y-T, Lin Y-T, Chen C-Y & Eskalen A (2018) Two novel fungal symbionts *Fusarium kuroshium* sp. nov. and *Graphium kuroshium* sp. nov. of Kuroshio shot hole borer (*Euwallacea* sp. nr. *fornicatus*) cause Fusarium dieback on woody host species in California. *Plant Disease* **102**: 1154-1164.

Paap T, De Beer ZW, Migliorini D, Nel W & Wingfield MJ (2018) The polyphagous shot hole borer (PSHB) and its fungal symbiont *Fusarium euwallaceae*: a new invasion in South Africa. *Australasian Plant Pathology* **47**: 231-237.

Ploetz RC, Hulcr J, Wingfield MJ & De Beer ZW (2013) Destructive tree diseases associated with ambrosia and bark beetles: black swan events in tree pathology? *Plant Disease* **97**: 856-872.

Roossinck MJ (2015) Metagenomics of plant and fungal viruses reveals an abundance of persistent lifestyles. *Frontiers in Microbiology* **5**: 767.

Summerell BA, Salleh B & Leslie JF (2003) A utilitarian approach to *Fusarium* identification. *Plant Disease* **87**: 117-128.

Umeda C, Eskalen A & Paine TD (2016) Polyphagous shot hole borer and Fusarium dieback in California. *Insects and Diseases of Mediterranean Forest Systems* **6*:*** 757-767. Springer.

van den Berg N, du Toit M, Morgan S, Fourie G & de Beer ZW (2019) First report of *Fusarium euwallaceae* causing necrotic lesions on *Persea americana* in South Africa. *Plant Disease* **10**: 10-18-1818-PDN.

Final Summary

The polyphagous shot hole borer (PSHB) or *Euwallacea fornicatus* is an ambrosia beetle, originally from South-East Asia, that can cause extensive damage, to a large variety of trees, by acting as a vector to inoculate its pathogenic fungal partner, *Fusarium euwallaceae,* directly into host plants, causing Fusarium Dieback (FD) (Eskalen *et al.*, 2012, Freeman *et al.*, 2013). In a recent study in South Africa, this ambrosia beetle-fungal complex was detected, damaging *Platanus x acerifolia* (London Plane) trees (Paap *et al.*, 2018). This observation caused concern to local agriculture, particularly to avocado growing communities, as this invasive beetle is internationally notorious for the damage it has caused specifically to avocado, in Israel (Mendel *et al.*, 2012) and California, in the United States of America (Rugman-Jones & Stouthamer, 2016).

This study was therefore commenced to survey, monitor and identify the beetles associated with disease on avocado and to identify fungal pathogens/symbionts connected to beetle-associated damage on avocado plants, in South Africa. During this investigation, the presence of several wood-boring beetles and fungi associated with damage to avocado were detected. Alongside several ambrosia beetles and ambrosial fungi that were detected, the causal agent for FD (*F. euwallaceae*) was detected causing damage to a residential avocado tree, in Johannesburg, in Gauteng. Molecular and phylogenetic identification confirmed the isolate's identity as being genetically identical to the isolates from avocado, in Israel and California (Mendel *et al.*, 2012, Freeman *et al.*, 2013, O'Donnell *et al.*, 2016, Na *et al.*, 2018). A subsequent pathogenicity trial fulfilled Koch's Postulates and confirmed the isolate's pathogenicity to avocado. This finding was submitted for publication in The American Phytopathological Society Journal: Plant Disease - 10-18-1818-PDN. The impact of this discovery emphasises the introduction of this pest complex and indicates

significant geographic spread from where it was first detected in South Africa, in Pietermaritzburg, in KwaZulu-Natal. This intention of this research was to highlight the threat posed by ambrosia beetles, particularly the PSHB, to local agriculture, specifically to that of the avocado industry as well as the biodiversity of native and indigenous plants.

In South Africa, a lack of previous attention to beetle damage means that currently-applied agricultural practices are unequipped and uninformed to detect, diagnose and treat the disease and to prevent its spread. Therefore, this study also performed investigations towards trying to combat FD. This included screening the susceptibility of two avocado fruit cultivars, to two fungal symbionts associated with the PSHB. This study found evidence that suggests standalone pathogenicity for the two symbionts (*F. euwallaceae* and *Graphium euwallaceae)*, that were assessed, to both cultivars and also found that a co-inoculation of the two symbionts resulted in substantially more severe disease symptoms than when inoculated on their own. These findings indicate the complexity of this disease and the importance of studying the overall microbial diversity associated with vector organisms, such as ambrosia beetles, towards better understanding the interactions between the organisms involved in the causation of disease.

Additionally, the efficacy of different chemical and biological control agents, against *F. euwallaceae,* were assessed. *In vitro* assays proved that all of the chemical and biological agents that were tested in this study, indicated promising prospects, as their action led to a significant decrease in the growth of *F. euwallaceae* mycelia. For two of the chemical agents, complete mycelial inhibition was observed. However, when comparing the sensitivity of these agents to what is observed *in planta* (Mayorquin *et*

al., 2018), to the results from this study, underestimations and limitations of effective *in vitro* concentrations were emphasised. These findings contribute towards the management of FD, by indicating increased pathogenicity of the combined effects of the fungal associates of the PSHB and also addresses the high demand for the consideration and application of potential chemical and biological agents for the treatment of FD, in South Africa.

The findings and conclusions from this thesis provide a basis for the further study of the PSHB and other ambrosia beetles, in South Africa, particularly on avocado. Research towards ambrosia beetles should be aimed at developing existing knowledge and contributing towards sustainable, effective and holistic management systems.

REFERENCES

Eskalen A, Gonzalez A, Wang D, Twizeyimana M, Mayorquin J & Lynch S (2012) First report of a *Fusarium* sp. and its vector tea shot hole borer (*Euwallacea fornicatus*) causing Fusarium dieback on avocado in California. *Plant Disease* **96**: 1070-1070.

Freeman S, Sharon M, Maymon M, Mendel Z, Protasov A, Aoki T, Eskalen A & O'Donnell K (2013) *Fusarium euwallaceae* sp. nov.—a symbiotic fungus of *Euwallacea* sp., an invasive ambrosia beetle in Israel and California. *Mycologia* **105**: 1595-1606.

Mayorquin JS, Carrillo JD, Twizeyimana M, Peacock BB, Sugino KY, Na F, Wang DH, Kabashima JN & Eskalen A (2018) Chemical management of invasive shot hole borer and Fusarium dieback in California sycamore (*Platanus racemosa*) in southern California. *Plant disease* **102**: 1307-1315.

Mendel Z, Protasov A, Sharon M, Zveibil A, Yehuda SB, O'Donnell K, Rabaglia R, Wysoki M & Freeman S (2012) An Asian ambrosia beetle *Euwallacea fornicatus* and its novel symbiotic fungus *Fusarium* sp. pose a serious threat to the Israeli avocado industry. *Phytoparasitica* **40**: 235-238.

Na F, Carrillo JD, Mayorquin JS, Ndinga-Muniania C, Stajich JE, Stouthamer R, Huang Y-T, Lin Y-T, Chen C-Y & Eskalen A (2018) Two novel fungal symbionts *Fusarium kuroshium* sp. nov. and *Graphium kuroshium* sp. nov. of Kuroshio shot hole borer

(*Euwallacea* sp. nr. *fornicatus*) cause Fusarium dieback on woody host species in California. *Plant Disease* **102**: 1154-1164.

O'Donnell K, Libeskind-Hadas R, Hulcr J, Bateman C, Kasson MT, Ploetz RC, Konkol JL, Ploetz JN, Carrillo D & Campbell A (2016) Invasive Asian *Fusarium–Euwallacea* ambrosia beetle mutualists pose a serious threat to forests, urban landscapes and the avocado industry. *Phytoparasitica* **44**: 435-442.

Paap T, De Beer ZW, Migliorini D, Nel W & Wingfield MJ (2018) The polyphagous shot hole borer (PSHB) and its fungal symbiont *Fusarium euwallaceae*: a new invasion in South Africa. *Australasian Plant Pathology* **47**: 231-237.

Rugman-Jones P & Stouthamer R (2016) Polyphagous-and Kuroshio shot hole borers: invasive *Euwallacea* spp. threatening Californian agriculture and natural areas. *Laurel Wilt Summit, UF IFAS Tropical Research and Education Center* 3-4.