Assessment of the pathogenicity of *Fusarium euwallaceae* to grapevine and deciduous fruit trees in South Africa and its rapid detection in woody tissues

by

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DECLARATION

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SUMMARY

A myriad of different tree species in South Africa are under threat from the invasive woodboring beetle, *Euwallacea fornicatus* (Coleoptera: Curculionidae: Scolytinae), the polyphagous shot hole borer, PSHB. While constructing galleries in the wood of hosts these beetles release spores of a mutualistic fungus, *Fusarium euwallaceae* (Hypocreales; Nectriaceae).The fungus colonises the xylem tissues and acts as the primary food source for the beetle, but colonisation can lead to Fusarium dieback disease in susceptible hosts. Many economically important fruit tree species have been identified as possible hosts; however, no assessments have specifically tested the pathogenicity of *F. euwallaceae* towards these. The work presented in this thesis set out to evaluate the susceptibility of different deciduous fruit trees (plum, nectarine and apple) and grapevine to *F. euwallaceae*. The effect of branch diameter and increased inoculum load on the rate of lesion development was also assessed. *Fusarium euwallaceae* was pathogenic to all fruit tree species and cultivars tested but no evidence of disease development was recorded in grapevine. There were no significant differences in virulence between the different isolates. There was also no evidence that differences in branch diameter or differences in the number of inoculation points on a branch can affect the growth rate of *F. euwallaceae*.

For monitoring, the presence of PSHB is often determined by positive identification of *F. euwallaceae* without collection of the beetle, particularly when beetles are not able to establish viable colonies. Standard approaches to identify *F. euwallaceae* are costly and labour- and time intensive as the fungus needs to be isolated and cultivated from freshly collected material, the DNA needs to be extracted and purified, and the DNA needs to be sequenced for a marker that can be used to identify it. In this thesis a faster, more accessible, and cheaper tool for the identification of *F. euwallaceae* in both fresh and dried wood samples which increases monitoring capacity when resources are limited is presented. A species-specific marker was identified from literature and an optimized PCR protocol for the identification of *F. euwallaceae* was developed that removes requirements to rear the fungus, purify its DNA and to sequence a DNA marker. This protocol was tested on eleven different hosts, all but one of which that produced positive results in at least one of the replicates. Amplification was not possible in one of the hosts likely due to the high concentration of PCR inhibitory compounds. In cases like this, a secondary measure based on the protocol developed here can be used where fungal isolates are first obtained from diseased woody tissues and these subjected to the rapid detection protocol. Amplification of *F. euwallaceae* DNA using this approach had a 100% reproducibility rate.

OPSOMMING

'n Magdom verskillende boomspesies in Suid Afrika is bedreig deur die indringer stamboorder, *Euwallacea fornicatus* (Coleoptera: Curcilonidae: Scolutinae), die polifage stompkopboorder , (PSHB). Terwyl die kewer gate deur die hout boor van gashere stel dit spore van sy mutualistiese swam, *Fusarium euwallaceae* (Hypocreales; Nectriaceae), vry. Die swam koloniseer die xileem weevsel en dien as die primêre voedingsbron vir die kewer, maar kolonisering kan lei tot Fusarium afsterwe siekte in vatbare gashere. Baie boom spesies wat ekonomies belangrik is, is al identifiseer as moontlike gashere; maar geen assesserings wat spesefiek die patogenisiteit van *F. euwallaceae* op hierdie gashere toets was al uitgevoer nie. Die werk wat in hierdie tesis aangebied word stel om die vatbaarheid van verskillende sagtevrugtebome (pruim, nektarien en apple) en wingerdstokke aan *F. euwallaceae* te evalueer. Die effek wat tak diameter en toegeneemde inokulum lading het op die koers van letsel groei was ook geassesseer. *Fusarium euwallaceae* was patogenies tot alle vrugteboom spesies en kultivars wat getoets is, maar geen bewyse van siekte ontwikkeling was waargeneem in wingerd nie. Daar was geen verskille in virulensie tussen verskillende isolate gevind wat betekenisvol was nie. Daar was ook geen bewyse dat verskille in tak diameter of verskille in die nommer van inokulasiepunte die koers van *F. euwallaceae* groei affekteer nie.

Vir monitering, is die teenwoordigheid van PSHB vas gestel deur positiewe identifiikasie van *F. euwallaceae* sonder versameling van die beesie, veral wanneer beesies nie lewensvatbare kolonies vestig nie. Standaard benaderings om *F. euwallaceae* te identifiseer is duur en arbeids- en tyd intensief omdat die swam eers geisoleer en gekweek moet word vanaf vars versamelde plant material, dan word DNA ekstraksie uitgevoer en DNA word gesuiwer. Daarna moet volgordebapaling uitgevoer word met ‘n merker wat kan gebruik word om *F. euwallaceae* te identifiseer. In hierdie tesis word ‘n vinniger, meer toeganklikke en goedkoper metode vir die identifikasie van *F. euwallaceae* in beide vars en gedroogte plantmateriaal aangebied. ‘n Spesies-spesefieke merker was identifiseer vanaf literatuur en ‘n geoptimiseerde PCR protokol was ontwikkel vir die identifikasie van *F. euwallaceae* wat die vereistes verwyder om die swam eers te kweek dan DNA ekstraksie- en DNA merker volgordebepaling uit te voer. Hierdie protokol was getoets op elf verskillende gashere, waarvan almal behalwe een positiewe resultate in ten minste een van vyf replikate positiewe resultate gegee het. Versterking was nie moontlik in een van die gashere nie, waarskynlik omdat daar hoë konsentrasies van inihiberende verbindings teenwoordig is. In gevalle soos hierdie is daar ‘n sekondêre benadering wat gebasseer is op die protokol wat hier ontwikkel is; die vinnige opsprong protokol kan toegepas word op swam isolate wat vooraf verkry is vanaf siek houtagtige weevsels. Versterking van *F. euwallaceae* DNA met hierdie

benadering het ‘n 100% reproduseerbaarheid.

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CONTENTS

CHAPTER 1:

General Introduction

Bark- and ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) act mainly as decomposers of dead or weakened shrubs and trees in native habitats (Li *et al.*, 2016; Stouthamer *et al.*, 2017). These have diversified tremendously (currently ca. 3,400 species described) and evolved adaptations to varied food sources, hosts and even specific host organs (Farrell *et al*., 2007). Bark beetles, being phloeophagous, primarily feed on dead or dying phloem tissues beneath the bark of woody plants. They often associate with various fungi that could aid in their nutrient acquisition (Whitney, 1982). Ambrosia beetles are often xylomycetophagous, and construct galleries deep within sapwood and hardwood of trees (Beaver, 1989). Even though they burrow galleries within the host, many species do not feed on the nutrient-poor host xylem tissues. Instead, they rely on symbiotic fungi to establish in the galleries and feed on their fungal gardens as a means of nutrient acquisition (Batra, 1963).

Because of their cryptic habits and associations with dead and dying trees, ambrosia beetles are often accidentally transported to new environments and countries as passengers on or within wooden products (Meurisse *et al*., 2019). In their native habitats ambrosia beetles rarely attack healthy trees. However, in invaded habitats they may infest apparently healthy hosts and their mutualistic fungi may be pathogenic to these living trees (Ploetz *et al*., 2013). Many species pose significant threats to indigenous, agricultural and urban trees around the world (Ploetz *et al*., 2013). For example, the causal organism of Laural wilt, *Raffaelea lauricola,*(Ophiostomatales; Ophiostomataceae) is vectored by the Asian ambrosia beetle, *Xyleborus glabratus* and has led to the losses of over 300 million redbay (*Persea borbonia*) trees in south-eastern United States since the early 2000`s (Hughes *et al*., 2017). Some species of ambrosia beetles, such as those belonging to the *Euwallacea fornicatus* (Eichoff) species complex (Coleoptera: Curculionidae: Scolytinae), have been reported to also attack healthy trees in the native region of the species complex (Coleman *et al.,* 2019; Gomez *et al.,* 2019). The ambrosia beetle, *Euwallacea fornicatus* Eichhoff (Coleoptera: Curculionidae: Scolytinae), together with its fungal symbiont *Fusarium euwallaceae* (Hypocreales; Nectriaceae) cause Fusarium dieback of susceptible hosts (Eskalen *et al*., 2013; Freeman *et al*., 2013). Commonly referred to as the polyphagous shot hole borer (PSHB), *E. fornicatus* has been recorded in 207 tree species in the USA including those that occur in urban landscapes, native tree species as well as trees from agricultural landscapes (Eskalen *et al*., 2013). In Israel, PSHB has been reported to attack 52 tree species (Mendel *et al*., 2017) and in South Africa it has been detected to infest, or attempt to infest, at least 130 tree species (Van Rooyen

et al., 2021). Recent introduction into western Australia has also been confirmed but PSHB has seemingly not infested many host species yet (IPPC, 2021).

In its native regions in Southeast Asia i.e., Thailand, Vietnam, China and Taiwan, *E. fornicatus* colonises stressed or dying hosts (Li *et al*., 2016; Stouthamer *et al*., 2017), however, it has also been reported to attack healthy trees in its native region (Coleman *et al.,* 2019; Gomez *et al.,* 2019). At present, non-native countries where PSHB has been recorded or has invaded are Israel, the United States of America, South Africa, Poland, Italy, Germany, Netherlands, and Australia (Mendel *et al*., 2012; Eskalen *et al*., 2012; Paap *et al*., 2018; IPPC, 2021, Schuler *et al.,* 2021). In the invaded regions, PSHB has caused immense damage in Israel, the USA and South Africa (Eskalen *et al*., 2012; Mendel *et al*., 2012; Paap *et al*., 2018; Van Rooyen *et al*., 2021). In terms of agriculture, PSHB can reproduce in avocado trees and has become a threat to avocado production both in the USA and Israel (Mendel *et* al., 2012; Eskalen *et al*., 2013). Other commercially produced crops that the beetle or its fungus has been isolated from include pecan nuts, grapevine, citrus, macadamia nut, various stone fruit and olive (Van Rooyen *et al*., 2021). Most of these reports stem from observations of trees planted in gardens. Major impacts of PSHB infestations of these have not yet been reported either because the beetle cannot breed in these hosts, the fungus is not pathogenic to these or PSHB has not yet reached major production areas of these hosts. Investigating the possible impact of PSHB on these crops is therefore an urgent research priority. The work presented here focuses on determining the pathogenicity of *Fusarium euwallaceae* on commercially produced deciduous fruit trees in the Western Cape province of South Africa as a first step in assessing possible future impacts of the PSHB invasion. A strong future need for diagnostics to identify infestations is envisaged and the development of a rapid diagnostic tool for the identification of *F. euwallaceae* is also investigated.

Taxonomic history of PSHB

The *E. fornicatus* species complex consists of *ca*. four ambrosia beetle species naturally distributed mostly in Asia Smith *et al*., 2019). The genus comprises small beetles (*ca*. 2 mm in length) (Figure 1) that often infest stressed host tress in their native environments. Many species are morphologically very similar which makes identification difficult, especially for non-specialists. For example, members of the *E. fornicatus* species complex are morphologically nearly identical, which has caused much confusion in previous research and management studies (Gomez *et al*., 2018). Each species in the complex, while morphologically similar, are different in terms of their respective fungal symbionts, geographic distribution, and host ranges (Freeman *et al*., 2013, Gomez *et al*., 2018; Na *et al*., 2018). The tea shot hole borer (TSHB, now *Euwallacea perbrevis*), originally also referred to as *E. fornicatus,* cause major damage to

tea (*Camelina sinensis*) plantations in Sri Lanka (Danthanarayana. 1968). Beetles responsible for damage caused on various trees in California (USA) (Rabaglia *et al*., 2006) were also initially identified as the TSHB (Mendel *et al*., 2012). Populations discovered on trees in Israel were morphologically indistinguishable from the TSHB (Mendel *et al*., 2012), but these were genetically distinct from those found in Sri Lanka and initially referred to as *Euwallacea* sp. near *fornicatus* (O'Donnell *et al*., 2015; Stouthamer *et al*., 2017). These were genetically identical to the populations first collected in the USA and was then later described as the polyphagous shot hole borer (PSHB, *E. fornicatus*) (Eskalen *et al*. 2013). Gomez *et al*. (2018) were able to address the ambiguity of the *E. fornicatus* species complex with a gene-based approach to resolve issues with the overlapping morphological characters, using samples from additional geographic locations. Four main phylogenetic clades of the species complex were resolved, and each given a new name; *Euwallacea whitfordiodendrus* (polyphagous shot hole borer [PSHB]), *Euwallacea fornicatus,* (tea shot hole borer clade a [TSHBa]), *Euwallacea fornicatior* (tea shot hole borer clade b [TSHBb]), and *Euwallacea kuroshoi* (kurushio shot hole borer [KSHB]) (Gomez *et al*., 2018). The species complex was revaluated after the rediscovery of a lost syntype (Smith *et al*., 2019) and the resulting taxonomic changes of the species names proposed were *Euwallacea fornicatus* (Eichoff)(PSHB), *Euwallacea Perbrevis* (Schedl)(TSHBa), *Euwallacea fornicatior* (Eggers)(TSHBb), and *Euwallacea kuroshio* (Gomez and Hulcr)(KSHB) (Smith *et al*., 2019).

Fungal symbioses and biology of PSHB

Foundress beetles initiate a new colony by burrowing into the xylem of the host (in sapwood) where spores of fungal symbionts are released during excavation from specialised structures located at the base of the mandibles known as oral mycangia (Hulcr *et al*., 2007; Fraedrich *et al*., 2008; Kasson *et al*., 2013). These fungi colonise the nutrient-poor xylem and serve as a means of nutrient acquisition for the beetle colony (Farrell *et al*., 2007). PSHB and TSHBa show strict preference for their respective fungal symbionts (Freeman *et al*., 2013), however the relationship of different species of the *E. fornicatus* species complex to other fungal symbionts of ambrosia beetles is likely promiscuous in its native region (Carrillo *et al*., 2019; Lynn *et al*., 2021). The primary nutritional fungal symbiont associated with adult PSHB in invaded regions is *Fusarium euwallaceae* (Hypocreales; Nectriaceae). AF-2, *F. euwallaceae*, forms part of the Ambrosia *Fusarium* Clade (AFC), a clade of *Fusarium* spp. exclusive to ambrosia beetles (Kasson *et al*., 2013; Freeman *et al*., 2016). Other *Fusarium* spp. associated with PSHB identified by Carillo *et al*., (2019) in its native region were AF-12 (*Fusarium kuroshium* [Hypocreales; Nectriaceae]), AF-13, AF-14, AF-15, AF-16, AF-17 and AF-18. Two other fungal species, *Graphium euwallaceae* (Microascales; Microascales incertae sedis) and

Paracremonium pembeum,(Hypocreales; Nectriaceae) are also associated with PSHB (Lynch *et al*., 2016). It is thought that *G. euwallaceae* may serve as an additional food source for the beetle and its larvae (Freeman *et al*., 2016). The nature of the symbiosis of *P. pembeum* with PSHB remains unclear (Freeman *et al*., 2016). However, the fungal species most isolated from infested hosts and mature adult female oral mycangia in invaded regions remains *F. euwallaceae,* which suggests that it plays a crucial role in establishing fungal galleries in new hosts (Freeman *et al*., 2016; Lynch *et al*., 2016).

The mode of sex determination of many ambrosia beetles including that of PSHB is based on a haplo-diploid system in which unmated females lay haploid/unfertilized eggs that develop into male beetles for the purpose of reproducing with either the foundress female or siblings (Cooperband *et al*., 2016). After sexual reproduction, diploid/fertilized eggs will develop into females (Cooperband *et al*., 2016). Very few mated females produce only female offspring in a single colony, likely to encourage outbreeding for a small portion (14%) of the population (Cooperband *et al*., 2016). The small males are not capable of flight and therefore do not play a role in the dispersal and colonization of new hosts. Females produce *ca*. 32 fertilised eggs that can develop into adults after 22 days (Cooperband *et al*., 2016). Reproduction of PSHB is therefore very rapid and populations can increase exponentially when in optimal habitats. This increases their chances for spread and establishment into new environments (Van Rooyen *et al*., 2021).

Many forms of spread are possible for PSHB. It can travel locally by flight at roughly 10-20 km/year as had been observed in Israel (Leathers, 2015). Spread internationally and over large distances within a country can be attributed to infested wood and wood packaging material which is considered one of the most important ways in which exotic Scolytinae spreads (Faulkner *et al*., 2016; Meurisse *et al*., 2019). Movement through infested firewood, pruned branches that had not been treated, and infested nursery stock is also very possible (Leathers, 2015; Meurisse *et al*., 2019). This makes curbing the spread of PSHB very difficult, especially in developing countries like South Africa where the informal wood trade is an important resource for the livelihoods of many citizens (Guild and Shackleton, 2018).

PSHB in South Africa and in the Western Cape Province in particular

In 2016, the South African National Biodiversity institute launched a sentinel forest project, which led to the first official report of PSHB in South Africa in the KwaZulu-Natal province from the National Botanical Gardens in Pietermaritzburg (Paap *et al*., 2018). PSHB had since been detected in every province of South Africa except Limpopo. A preliminary list includes both native and non-native host species of PSHB in South Africa, as well as economically important crops that occur throughout the country (FABI, 2021). Van Rooyen *et al*., (2021) has since

identified 130 hosts of PSHB in South Africa, of which 1000 individual trees in Cape Town urban areas have been infested and will cost the city *ca.* ZAR 3 million to remove. The cost of replacement of these trees have been estimated to be ZAR 4.5 million. The impact of beetle infestation in native areas of South Africa is under investigation (Van Rooyen *et al*., 2021). Important crops produced in large production areas in the Western Cape province that are on this list include citrus, stone fruit, and grape. The occurrence of beetle attack on apple had been observed by Eskalen *et al*. (2013) and apple and cherry trees in garden settings in George in the Western Cape Province had recently been reported (van Rooyen *et al*., 2021). It is not possible to predict the potential economic losses that could result from PSHB establishment in large deciduous fruit production systems based on infestation reports of single trees in backyard settings such as these, and it is therefore necessary to investigate the activity of the beetle and its fungal symbiont in commercial orchards.

The Mediterranean climate of the coastal areas of the Western Cape is ideal for the development of PSHB colonies. The development temperatures for the beetle range from 13.34°C to 33.08°C with an optimum at 27.51°C (Umeda and Paine, 2019). Development slows below 18°C and progeny is unable to complete development at lower than 16°C (Umeda and Paine, 2019). Suitable temperatures for beetle development in the province therefore occur year-round with only few weeks/months in winter when development would not be possible. It is therefore no surprise that the beetle was able to rapidly colonise trees and spread *ca*. 6 km from its putative point of introduction in Somerset West, Cape Town, South Africa in as little as two years (Roets, unpublished data). The beetle has now spread to areas that are surrounded by vineyards and deciduous fruit tree orchards, necessitating urgent determination of possible future impacts.

Symptoms

PSHB has a vast host range and symptom expression among different host species vary. Common symptoms associated with PSHB infestation include gumming at entry holes, staining of vascular tissues surrounding galleries and sugar exudation or “sugar volcanoes” at entry holes (Eskalen *et al*., 2013). Establishment of *F. euwallaceae* in hosts does not guarantee colonisation or aggressive attack by the beetle, since cases of *F. euwallaceae* establishment are often observed without beetle reproduction (Mendel *et al*., 2017). Also, PSHB does not seem to discriminate between hosts that would be good for colony establishment and those that may not (Mendel *et al*., 2021). To discriminate between the various types of hosts, scientists classify these as hosts in which 1) the fungus can establish, and beetle can reproduce, 2) those in which the fungus can establish but the beetles cannot reproduce and 3) those that are not suitable for either the beetle or the fungus. Susceptibility to *F. euwallaceae* paired with high

levels of beetle activity within hosts can cause dieback symptoms, and in some cases, tree mortality (Eskalen *et al.,* 2013). This because *F. euwallaceae* colonizes vascular tissues, which blocks transport of water and nutrients to upper parts of the stem or branch (Mendel *et al*., 2012; Eskalen *et al*., 2012; Eskalen 2013). Symptoms of Fusarium dieback include branch and foliar wilting, vascular discolouration, foliar discolouration and weakened branches (Mendel *et al*., 2012). Together, the activity of the beetle and the growth of the fungal symbiont may significantly reduce productivity of crops and may cause plant death in highly susceptible hosts (Eskalen *et al*., 2012). In terms of agricultural crops, these symptoms and impacts have mostly been realised on avocado (Mendel *et al*., 2017; Eatough Jones and Paine, 2017). Some hosts experience only superficial attack while others are heavily colonised, and the mechanisms surrounding host-pest interactions remain elusive (Eskalen *et al*., 2013). Mendel *et al*. (2017) reported that sites on individual trees that were previously colonised by PSHB are more likely to undergo further attempts. This may be due to fungal or plant volatiles that the beetles associate with weakened or damaged tissues that act as attractants (Hulcr *et al*., 2011; Mendel *et al*., 2017). However, other factors such as having highly infested trees in the immediate surrounding area will also contribute to increased beetle attack events. Mendel *et al*., (2021) characterized three main groups of hosts for *E. fornicatus*; (i) those which are not susceptible to the fungal symbiont, (ii) those which slow the development of *F. euwallaceae*, and (iii) those that are very susceptible to *F. euwallaceae* and allow the fungus to proliferate. It was also suggested by Mendel *et al*., (2021) that the susceptibility of a tree to *F. euwallaceae* determines the suitability of the host for PSHB reproduction, however, the beetle itself does not distinguish between reproductive and non-reproductive hosts. Lynch *et al*. (2021) has suggested that hosts are either competent, non-competent or killed-competent, i.e., that a host is either able to support beetle reproduction, is unsuitable for reproduction or is killed by the beetle and pathogen. However, incompetent hosts can still be susceptible to *F. euwallaceae* colonisation without the establishment of beetle colonies (Lynch *et al*., 2021). Interestingly, susceptibility has been proven to not be entirely random to certain species. Competent hosts and non-competent hosts appear to be less conserved within lineages than killed-competent hosts, indicating strong relationship between phylogeny and susceptibility of hosts (Lynch *et al*., 2021). For example, a large portion (78%) of tree species of the Rosids clade studied by Lynch *et al*., (2021) are killed-competent hosts. This group includes the Rosaceae to which many fruit trees such as pome fruit, stone fruit and almonds belong.

Management

Many aspects of PSHB`s ecology provide challenges when it comes to developing sustainable management practices. Because of the haplo-diploid mode of reproduction and already mated

females leaving galleries in hosts, they do not require sex pheromones to use as chemical lures. Quercivorol, an aggregation pheromone of some other Scolytinae beetles also attracts PSHB and is commonly used in monitoring efforts (Dodge *et al*., 2017). Byer's *et al.,* (2017) determined that the 1X release of quercivorol lure's attractiveness to the beetle is similar to that of an avocado branch. In combination with repellents, piperitone and verbenone, quercivorol can be used in mass trappings using a push-pull method (Byer's *et al.,* 2020). This is however costly and the main approach to survey and detection is through visual inspections. By pruning infested branches and removing heavily infested trees it is possible to reduce future population increase by inhibiting future mass-colonisation events. However, removal of large numbers of branches and whole trees is highly undesired in fruit tree production systems. Infested materials should be disposed of appropriately to avoid possible transmission between sites and re-introduction into orchards. Options for disposal of infested material include chipping, solarization and burning. Material that is chipped to less than 5 cm and solarised thereafter seems most practical (Eatough Jones and Paine, 2015). Another cultural control method that may be used in orchards is the planting of species in and around orchards which do not support beetle breeding and that will not contribute to increased propagule pressure. Coupled to this, planting of varieties that are less susceptible to the fungus and the beetle is advised, but screenings still need to be conducted to find more resistant cultivars.

Chemical control options such as systemic insecticides which have previously provided some success on other wood borers (Mayorquin *et al*., 2018), may also be effective against PSHB. However, because PSHB beetle`s primary food source is fungi, it may remain largely unaffected by the presence of systemic insecticides. It may be possible to instead apply a systemic fungicide such as propiconazole (Mayorquin *et al*., 2018) which will inhibit colonisation of fungal symbionts and in turn prevent establishment of beetle colonies, however the efficacy is still under investigation in South Africa (Nependa, unpublished data). A potential short-term solution utilising a combination of systemic- and contact insecticides as well as a chemical fungicide on low to moderately infested trees may provide some control options given that the pesticides are applied as a preventative measure or in early stages of infestation (Eatough Jones *et al*., 2018).

Future use of potential biological control agents may play an important role in the management of PSHB. Sourcing candidates from the native region of PSHB may prove fruitful. Hymenoptera (Nierhaus-Wunderwald 1993) parasitism of the beetle has been observed in its native range in China by Li *et al*. (2016). However, prospects of biological control using natural enemies will not be viable for some time as e.g., studies in South Africa have just been initiated (Townsend, pers. comm.). A biological fungicide *Bacilus subtilis* has shown to provide possible short-term control, however as with the proposed chemical control options, its efficacy may be

heavily dependent on timeous application during early stages of attack by the beetle (Eatough Jones and Paine, 2018).

Economic impact

The vast host range of PSHB presents potential negative economic impact in many environments, such as urban, native, and agricultural landscapes. Biological invasions, particularly by xylem- and phloem-inhabiting insects has led to as much as $1.7 billion in local government mitigation efforts in the United States (Aukema *et al*., 2011). Heavily infested trees around pedestrian walkways, parking lots and buildings pose a risk to persons and property. Dense gallery formation and development of dieback symptoms can weaken branches or trunks of large trees which could fall and cause destruction of property, and vehicles, and pose serious injury risk to pedestrians and motorists (Kovacs *et al*., 2011; Aukema *et al*., 2011). Established populations in urban landscapes may cost municipalities millions of dollars to deal with mitigation of these risks which is problematic in the South African context where development of infrastructure and maintenance of urban environments are already under severe strain (De Wit *et al*., 2021). It is estimated by De Wit *et al*. (2021) that the cost of PSHB invasion will be *ca*. $50 per South African citizen per year for the next 10 years. Should PSHB show high infestation potential in fruit orchards (in addition to avocado), the cost could be much greater. The revenue from fruit export in South Africa contributes significantly to the country's GDP. For apple, pear and stone fruit, the total sales price in 2020 were valued at ZAR 10 777.6 million, ZAR 4990.5 and ZAR 2671.4 million respectively (Hortgro, 2020). A total of 92 000 ha of vineyards exists in South Africa, of which 83% of production occurs in the Western Cape (SAWIS, 2021). *Fusarium euwallaceae* has previously been recovered from grapevine in George in the Western Cape (Van Rooyen *et al*. 2021) and from California (Eskalen *et al*., 2013). Wine and table grape production in the Western Cape contribute ZAR 55 billion to the national GDP of South Africa (SAWIS, 2021). However, because there is no data available of PSHB infestation and impacts in fruit and grape orchards/vineyards, the cost of potential losses is impossible to determine.

There are many ways in which PSHB and *F. euwallaceae* can cause yield losses in production systems. Occlusion of xylem vessels in the stem or branches reduces water and nutrient transport to foliage and fruit, causing wilt symptoms and having a negative effect on fruit set and/or fruit quality. Adopting current management practices may still contribute to a degree of yield loss. For example, in avocados where dieback symptoms have manifested, large scale pruning practices and potential tree removal will greatly reduce productivity as well as increase production costs (Leathers, 2015). Export fruit including table and wine grapes

generates a large portion of income in agricultural trade in South Africa, however trade may be impeded to countries that have listed PSHB as a quarantine pest.

Fusarium euwallaceae detection as a monitoring tool

Members of the *E. fornicatus* species complex associate with *Fusarium* spp., that belong to the Ambrosia Fusaria Clade (AFC) which forms a monophyletic clade within the *F. solani* species complex (FSSC) (Kasson *et al*., 2013; O`Donnell *et al*., 2015). Each member of the *E. fornicatus* species complex, normally associate with a different *Fusarium* species of the AFC (Hypocreales; Nectriaceae) (Cooperband *et al*., 2016). For example, the symbiont of *E. fornicatus* is *F. euwallaceae* (Lynch *et al*., 2016), whereas the symbiont of *E. kuroshio* is *Fusarium kuroshium* (Na *et al*., 2018). Both *F. euwallaceae* and *F. kuroshium* are causal organisms of Fusarium dieback in susceptible hosts (Eskalen *et al*., 2013; Na *et al*., 2018). The fungal symbiont of *E. perbrevis* (TSHB Clade a) is *F. ambrosium* while the symbiont of *E. fornicatior* (TSHB Clade b) remains unknown (Gomez *et al*., 2018; Smith *et al*., 2019). As mentioned previously, other *Fusarium* spp. have been detected that associate with the PSHB in Taiwan (Carillo *et al*., 2019). In addition, there are reports of other *Fusarium* species from Indonesia associated with TSHB (Lynn *et al*., 2021), however these have not been detected in invaded regions.

Timeous identification of PSHB beetles and diagnosis of Fusarium dieback in invaded regions is important for monitoring and management efforts. Presently, the general approach for fungal pathogen identification is to identify and describe symptoms, collect infected tissue, plate infected tissues onto media such as potato dextrose agar, carnation leaf agar or nutrient poor agar, allow cultures to grow, purify cultures and identify the taxa using colony characteristics and morphology (Summerell *et al*., 2003). This approach requires considerable time and colony characteristic analyses for identifications require skilled labour. Isolating microorganisms onto media can also often lead to misidentification where contamination of cultures has occurred during the isolation process. Additionally, morphological identification of many species, including the different *Fusarium* species associated with members of the *E. fornicatus* species complex is futile, since there are no morphological differences between these. Molecular identification procedures offer a faster approach to fungal identification. Typically, microbial cultures are used for DNA extraction for molecular identification using DNA sequence data of target genes (McCartney *et al*., 2003). Sequence data from the PCR products are obtained and compared to data available from online databases to match and identify the specific microorganism to genus or species level. This approach can however still be resource and time consuming, as some markers require high quality extracted DNA, and there is still the needed capacity to isolate, grow and store cultures. Where monitoring of invasive pathogens in

various regions are concerned, this option is therefore not always ideal. A rapid, species-specific diagnostic tool requiring only essential lab equipment, such as a PCR thermocycler and gel electrophoresis equipment, would aid monitoring efforts of PSHB in newly invaded regions. Freeman *et al*. (2013) demonstrated under laboratory conditions that PSHB has a strict preference for its fungal symbiont. Although there is evidence of fungal promiscuity of ambrosia beetles in their native region, this has not yet been observed in invaded regions (O'Donnell *et al*., 2015; Carrillo *et al*., 2019). Regardless, rapid diagnosis of Fusarium dieback where *F. euwallaceae* is the causal organism provides an indication of PSHB distribution which is paramount for monitoring purposes. In addition, colonisation of host tissue by *F. euwallaceae* in the absence of beetle colonies has often been observed, making monitoring that depends on the presence of PSHB colonies difficult, further necessitating a rapid detection method aimed at *F. euwallaceae* (Lynch *et al*., 2021; Mendel *et al*., 2021).

Various PCR markers for the identification of different *Fusarium* species have been developed (Baird *et al*., 2008; Visentin *et al*., 2009; Faria *et al*., 2012a, 2012b). The process for identifying candidate genes for diagnostic purposes can include two main pathways; by selecting genes which contain conserved regions for specific taxa, or by selecting species-specific genes through scanning the fungal genome (McCartney *et al*., 2003). Nucleic acid fingerprinting techniques such as those using restriction fragment length polymorphisms (RFLPs) and amplified fragment length polymorphisms (AFLPs) have also been used to produce highly specific markers in *Fusarium* (Botstein *et al*., 1980; Vos *et al*., 1995; Lievens *et al*., 2007). The process of DNA fingerprinting involves generating distinct DNA fragments from an organism which are then used as a source for genotypic information. Patterns of genetic markers generated in this way are then correlated to specific organisms. However, in highly speciose taxa such as the Ascomycetes, this approach would require a large amount of data from other species and strains of *Fusarium* in to validate the specificity of the markers (Lievens *et al*., 2007). A method using probe-based multiplex real-time PCR makes is available and can identify *F. euwallaceae* and distinguish it from other fungal symbionts associated with ambrosia beetles of the *E. fornicatus* species complex (Carillo *et al*., 2020). Species specific PCR markers have also been developed for *Fusarium* spp. of the *E. fornicatus* species complex which were achieved using comparative genomics and focusing on utilising protein coding genes that were species-specific (Short *et al*., 2017; Vázquez-Rosas-Landa *et al*., 2021). Short *et al*., (2017) identified a highly sensitive PCR marker for the identification of *F. euwallaceae* which was proven to be able to detect it in a multiplex reaction along with other *Fusarium* species of the Ambrosia Fusarium Clade (AFC). While the primer set designed by Short *et al*., (2017) showed high sensitivity and was able to detect fungal DNA extracted directly from beetle heads, it has not yet been tested in a direct PCR approach (PCR without purification of DNA from infected tissues/samples).

Few protocols exist that allow PCR of fungal DNA without prior DNA extraction and purification. Direct PCR can be difficult to achieve because polyphenolic compounds that are often present in wood and fungal tissues, can bind to DNA once oxidised, inhibiting the PCR reagent *Taq* polymerase (Peterson *et al*., 1997). Universal direct PCR protocols suitable for most biological samples have been described and these are able to produce quality PCR product that is sufficient to be sequenced, however these methods have not been tested on diseased woody tissue (Ben-Amar *et al*., 2017). These methods require minimal reagents, and it is possible that it could be utilised for detecting *F. euwallaceae* DNA directly from diseased woody tissues with some modifications, thereby expanding and simplifying monitoring efforts of PSHB.

Aims

The research presented in this thesis aims to assess the threat of the PSHB (*Euwallacea fornicatus*) and its fungal symbiont, *Fusarium euwallaceae,* to deciduous fruit trees and grapevine in South Africa, and to develop a direct PCR protocol for rapid and low-cost identification of *F. euwallaceae* in infected plants.

Objectives

1. Determine the susceptibility of grapevine and deciduous fruit trees to *Fusarium euwallaceae* in the Western Cape province of South Africa (Chapters 2 and 3).

2. Assess the effect of factors such as branch diameter, cultivar and number of inoculation points (as simulation of number of beetle infestation attempts) on host susceptibility to *F. euwallaceae* (Chapter 3).

3. Develop a PCR protocol for rapid detection of *F. euwallaceae* directly from diseased woody tissues (Chapter 4).

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Figure 1 : Illustration of *Euwallacea fornicatus*. Photo credit S.M. Smith

CHAPTER 2:

Assessment of the pathogenicity of *Fusarium euwallaceae* to grapevines in South Africa

Abstract

The introduction of the woodboring beetle *Euwallacea fornicatus* Eichhoff (Coleoptera: Curculionidae: Scolytinae) to new regions around the world has resulted in alarming impacts on trees in natural, urban, and agricultural landscapes. Successful colonization of susceptible hosts results in the proliferation of the beetle's fungal symbiont, *Fusarium euwallaceae* (Hypocreales; Nectriaceae) within the xylem tissues which can result in Fusarium dieback disease and plant death. *Euwallacea fornicatus* infestations of grapevine (*Vitis vinifera*) in gardens in South Africa was recently observed, but the susceptibility of this host species to Fusarium dieback disease is currently unknown. The purpose of the present study was to assess host response of two commercially grown grapevine cultivars to *F. euwallaceae* infection in South Africa's premier wine production region, the Western Cape province. Vines were inoculated with three isolates of *F. euwallaceae*, one of which was initially obtained from a vine in a garden and resulting lesion development was evaluated. Plants were inoculated using fungus-laden tooth picks inserted into small holes drilled into grapevine stems to simulate boring-beetle activity. Lesion development was monitored and *F. euwallacea* was re-isolated from inoculated tissues to fulfil Koch's postulates. Results indicated that *F. euwallacea* was unable to survive for longer than three months within inoculated tissues and that resulting lesion development was similar to that of the controls, indicating that *F. euwallaceae* is not a virulent pathogen of grape cultivars Merlot and Cabernet Sauvignon and when grown under dryland production conditions. As the fungus does not readily establish in these hosts, it is unlikely that *E. fornicatus* would pose a significant threat to grapevine production, but continued monitoring is needed as the vineyards studied here may not represent ideal conditions for *F. euwallaceae*. Additionally, *E. fornicatus* is potentially capable of acquiring new fungal symbionts in future which may be more virulent to grapevine.

Introduction

The plant pathogen *Fusarium euwallaceae* (Hypocreales; Nectriaceae) is vectored by *Euwallacea fornicatus* Eichhoff (Coleoptera: Curculionidae: Scolytinae), commonly referred to as the polyphagous shot-hole borer beetle (PSHB) (Eskalen *et al*., 2013). Together with the activity of the beetle, the fungal symbiont can cause Fusarium dieback disease in susceptible hosts (Eskalen *et al*., 2013; Freeman *et al*., 2013). In addition to native, ornamental, urban and

forest trees being threatened by PSHB, agriculturally important crops are also susceptible to PSHB infestation. In California and Israel, *Persea americana* (avocado) production has been the most severely affected by the introduction of PSHB (Eskalen *et al*., 2012; Mendel *et al*., 2012). Between 2009 and 2016, PSHB has spread to nearly every avocado production region in Israel, but its effect on other crops is understudied (Mendel *et al.*, 2017). In South Africa, PSHB has been reported from all but one province, having invaded native forests, urban trees, and backyards. In urban settings in Cape Town, over 600 trees have been identified for removal which will cost the City of Cape Town nearly ZAR 3 million (including the cost of monitoring) and a further ZAR 4.5 million to replace trees that will be removed (Van Rooyen *et al*., 2021). The effect of PSHB invasion on agricultural landscapes in South Africa is difficult to predict as most observations of infested hosts are from garden settings

PSHB is a woodboring beetle that constructs galleries within the xylem of woody hosts. During excavation, fungal spores are released from specialised structures known as mycangia which are located below the mandibles (Hulcr *et al.*, 2007; Fraedrich *et al.*, 2008). The spores germinate and grow into the xylem tissues of the host, blocking nutrient and water transfer in the plant that may lead to wilting and death (Eskalen *et al*., 2013). *Fusarium euwallaceae* (Hypocreales; Nectriaceae) is the primary food source of the beetle (Farrell *et al.*, 2007; Lynch *et al*., 2016), but other fungal associates of *E. fornicatus* have also been described by Lynch *et al.*, (2016) including, *Graphium euwallaceae* (Microascales; Microascales incertae sedis) and *Pacremonium pembeum* (Hypocreales; Nectriaceae). While the role of *G. euwallaceae* has been confirmed as a nutritional mutualist for different stages of beetle development, it has not been demonstrated to be pathogenic to hosts (Freeman *et al*., 2016). The role of *P. pembeum* as a mutualist or plant pathogen is not supported (Freeman *et al.*, 2016). The most important fungal species to PSHB survival appears to be *F. euwallaceae* as it is commonly isolated from the mycangia of foundress females, suggesting it has a fundamental role in establishing new colonies (Freeman *et al*., 2016). Typical external symptoms of beetle and *Fusarium* infestation into host trees include gumming, wood staining, white powdery exudates at entry holes, foliar wilting, discolouration and weakened branches (Mendel *et al*., 2012; Eskalen *et al*., 2013). Occlusion of xylem vessels by colonisation of *F. euwallaceae* can lead to restriction of water supply to upper plant parts, causing reduced fruit quality and great economic losses in production systems. PSHB can reproduce in certain hosts (so-called reproductive hosts) where new colonies are established (Eskalen *et al*., 2013). These hosts are of particular concern as they may suffer severe mortality rates.

In its native region of Southeast Asia, PSHB has been reported on dead or dying trees (Li *et al.*, 2016; Stouthamer *et al.*, 2017), although it has also been reported on healthy trees (Coleman *et al*., 2019; Gomez *et al*., 2019). In invaded regions, PSHB is often reported on hosts that appear healthy (Mendel *et al*., 2012; Eskalen *et al*., 2013; Paap *et al*., 2018; IPPC,

2021, Van Rooyen *et al*., 2021; Schuler *et al.,* 2021). The host range of the PSHB is vast, having been recorded on over 207 tree species in the USA and at least 52 in Israel (Mendel *et al.*, 2012; Eskalen *et al*., 2013). These lists include species from agricultural as well as natural and urban landscapes. Large scale economic losses have been recorded in avocado production in both the USA and Israel (Mendel *et al.*, 2012; Eskalen *et al*., 2013). The first report by Paap *et al*. (2018) of PSHB in South Africa came in 2017. It had since been reported in important crop species such as pecan, grapevine, citrus, macadamia, stone fruit, olive, and avocado trees (FABI, 2021). Currently, 130 tree species in South Africa have been identified with signs of beetle attack, where 48 of these are suitable for beetle reproduction and 82 are susceptible to *F. euwallaceae* (Van Rooyen *et al*., 2021). Preliminary data from survey plots in natural forests in George and Knysna showed that out of 2195 trees assessed for PSHB presence, 217 individuals were infested and 191 showed signs of beetle reproduction (Van Rooyen *et al*., 2021). Many reports come from individual plants in urban settings, making it difficult to determine the effects of Fusarium dieback in large scale production settings. The Western Cape province of South Africa is a major viticulture production area for both table grape and wine varieties, and the threat of the PSHB in viticulture production areas needs to be assessed as other ambrosia beetles have been reported as pests (Ruzzier *et al*., 2021).

The behaviour of the beetle suggests that it is rather opportunistic, and the success of beetle infestation is more likely affected by the susceptibility of a host to *F. euwallaceae* (Mendel *et al.*, 2021). When attacking hosts, PSHB does not differentiate between reproductive and non-reproductive hosts, however the beetle seems to preferentially attempt colonisation of trees that it perceives to be stressed (Hulcr and Dunn, 2011), due to emissions of fungal volatiles from damaged tissues (Hulcr and Dunn, 2011; Mendel *et al.*, 2017). Three categories of hosts for *F. euwallaceae* have been described by Mendel *et al*., (2021); (i) those which are resistant to *F. euwallaceae* colonisation, (ii) those that are able to slow the development of *F. euwallaceae,* and (iii) those that are highly susceptible to *F. euwallaceae*.

Differences in disease severity had been noted in different cultivars of avocado (Jones and Paine, 2017). This indicates that virulence of *F. euwallaceae* may also differ between cultivars of other host species and warrants further investigation. Lynch *et al*. (2021) studied the relationship between familial lineages of host species and susceptibility to *E. fornicatus* and *F. euwallaceae* and found that host susceptibility, particularly high susceptibility, is conserved within familial clades. The family Vitaceae, in which grapevine (*Vitis vinifera*) is taxonomically placed, was included in this assessment as a species that can support fungal growth but does not necessarily support beetle colonization (Lynch *et al*., 2021). The data used in this study included isolated reports from a botanical garden in Los Angeles, which potentially does not accurately represent the losses that could occur in commercial vineyards (Eskalen *et al*., 2013; Lynch *et al*., 2021). Characterisation of grapevine host status to *F. euwallaceae* would be

beneficial in developing appropriate management strategies for this crop as well as to provide opportunity for growers to take pre-emptive measures in curbing the potential impact of beetle.

The South African grapevine industry contributed ZAR 55 billion to the national GDP (Gross Domestic Product) and a total of ZAR 7.2 billion alone was contributed by the wine tourism industry in 2019 (SAWIS, 2021). Currently, 92 005 ha of wine grape production sites exist in South Africa where over 83% of production occurs in the Western Cape region (SAWIS, 2021). The grapevine industry in South Africa faces many challenges including the effects of climate change and pest introductions. PSHB was identified on grapevine by Eskalen *et al*., (2013) and a *F. euwallaceae* isolate have been recovered from grapevine found in George, a town located in the Western Cape region. Because of the rapid spread of PSHB throughout the country and cases of PSHB infestation reported in a wine grape growing area, Somerset West, in the Western Cape there is urgency to evaluate the risk of PSHB to grapevine production.

The aim of this study was to assess the threat of the PSHB fungal symbiont *F. euwallaceae* to selected grapevine cultivars in agricultural production landscapes. The objectives were (i) to determine the susceptibility of grapevine to *F. euwallaceae* and the growth rate of *F. euwallaceae* in *Vitis vinifera* and (ii) to determine the difference in cultivar susceptibility.

Materials and methods

Inoculum preparation

Isolates of *Fusarium euwallaceae* from three different sources (Table 1) were used in assessment of its pathogenicity towards selected grapevine cultivars. Bamboo wooden toothpicks that were 2 mm in diameter were autoclaved in a mixture potato dextrose agar (PDA) (Biolab, Midrand, South Africa) that was diluted to approximately 1/3 of the manufacturer's recommended concentration. This was done to saturate the toothpicks with growth medium and to encourage fungal growth throughout the wood. The agar-saturated toothpicks were then placed on petri dishes containing PDA. The selected isolates were inoculated onto petri dishes and allowed to incubate for 14-21 days at 25°C in the dark. PDA plates with agar-saturated toothpicks that were not inoculated with any fungal isolates served as controls.

Field trial

Two *Vitis vinifera* cultivars 'Merlot' and 'Cabernet Sauvignon' on the Nietvoorbij experimental farm in Stellenbosch, Western Cape province were used in trials. The plantation blocks were situated on the eastern side of a hill of approximately 70 m in elevation (33°54'05"S 18°52'34"E). These vineyards were not under irrigation. Vines were inoculated with fungi by drilling 1 cm deep holes in the main stem using a 2.5 mm diameter drill bit to mimic beetle

activity. Five holes were drilled into each stem, starting at *ca*. 10 cm from the point of branching of the cordons in a spiral-like fashion around the stem with each hole 5 cm from the previous one. Toothpicks colonised with *F. euwallaceae* were placed in each hole and subsequently sealed with Parafilm™ grating tape. Each individual vine was inoculated with a single isolate or control into all inoculation points. Twenty replicate plants per isolate or control that were chosen at random and at least 10 m from the vineyard edge, were inoculated. This was repeated for both cultivars. Therefore, a total of 80 plants per cultivar were included in the trial. Inoculated vines were left to incubate for one year after which the vines were to be harvested and lesion development within the xylem measured and plants scored for signs of dieback symptoms.

To assess possible disease development over time, an additional 30 replicates per isolate and control were inoculated for each cultivar. In this case vines only received a single hole for inoculation on the main stem, *ca*. 10 cm below the cordons. Ten replicate vines, chosen at random, were harvested after one, three-, and six-months post-inoculation and cut longitudinally through the inoculation points to measure lesion lengths (the length of the resulting stain in the xylem tissues) to assess the growth of *F. euwallacea*.

Isolations were made after each harvesting of plants to confirm *F. euwallaceae* as the causal organism of lesion development. This was done by cutting stems longitudinally to expose discolouration in the xylem presumably caused by the pathogen. Woody sections containing lesions were then surface sterilised in 70% ethanol for 1 min after which approximately 5 mm^3 stained tissue were plated onto PDA medium in petri dishes. These were then left to grow in the dark for approximately seven to 10 days and then morphologically evaluated to confirm the isolate to be that of *F. euwallaceae*.

Data analyses

All data were analysed using RStudio® Software (version 3.6.3; RStudio 153 Team, 2019). A Shapiro-Wilks test was used to determine whether data sets were normally distributed (Shapiro and Wilk, 1965). Data was subsequently assessed for homogeneity of variance using Levene`s test (Levene, 1960). Significance of differences in lesion length data was compared between cultivars, treatments and controls using Kruskal-Wallis tests (Kruskal, 1952), followed by Mann-Whitney U post hoc analyses when needed (Mann and Whitney, 1947). A probability level of 5% was considered significant.

Results

The first data generated in the present study was that of the of lesion development experiment in which first harvesting occurred after one month. Lesion development around inoculation points consisted of a dark stain that penetrated no more than 2 mm into the xylem from the

inoculation point. These same patterns were observed for controls and on both cultivars. Only three isolates of *Fusarium euwallaceae* could be recovered from Cabernet Sauvignon and four isolates from Merlot from all harvested material. There was no significant difference in lesion development between the isolates and with the controls in cultivars Merlot ($H = 3.1698$, $df = 3$, $p = 0.3662$), or Cabernet Sauvignon ($H = 3.181$, $df = 3$, $p = 0.3646$). The same pattern was revealed after three months post inoculation, with no further lesion development, however at this stage no *Fusarium* isolates were successfully recovered from any of the vines. This pattern was repeated for all further treatments and in all experiments. When lesion development was longer than 2 mm in any of the treated individuals, invariably fungal taxa other than *Fusarium euwallaceae* were recovered.

Discussion

This study aimed to determine the pathogenicity of *F. euwallaceae* on grapevine in South Africa. Results indicated that, even though the fungus can be reisolated after approximately a month post inoculation, it did not present any significant lesion development. After three months no further lesion development was observed, and re-isolation the fungus from any samples failed, indicating that the fungus could not survive within these tissues for prolonged periods. The same was seen in the other trials indicating that differences in cultivar did not increase pathogenicity, at least between the cultivars ‘Merlot’ and ‘Cabernet Sauvignon’ produced under these specific conditions. It was concludeg that *F. euwallaceae* is not pathogenic towards these grapevine cultivars and, in fact, other than for many other plant taxa it does not seem to survive in grapevine woody tissues for prolonged periods (Figure 1).

One of the isolates used in the present study originated from grapevine in George and it is likely that it was isolated soon after initial attack by the beetle. The beetle has not been recovered form grapevine and no reports of damage to grapevines have been reported from garden settings in South Africa. Eskalen *et al*. (2013) also recovered *F. euwallaceae* from grapevine from the Los Angeles Arboretum and the Huntington Library, Art Collections and Botanical Gardens in the Los Angeles County, and described grapevine as susceptible to Fusarium dieback. However, the plants were not destructively analysed for lesion development and symptoms were only scored based on exterior symptom expression (Eskalen *et al*., 2013). Isolations were made by extracting tissue only from PSHB entry holes, and not symptomatic tissue, to recover beetles and/or fungal isolates (Eskalen *et al*., 2013). The successful isolations of *F. euwallaceae* from beetle entry holes are likely due to remaining spores from colonisation attempts by the beetle. In the case of grapevine, only gumming was reported (Eskalen *et al*., 2013) which is not necessarily a symptom of fusarium dieback, but rather a symptom of attack or injury to the woody tissue of the host. No other external symptoms were noted nor was

reproduction recorded in grapevine by Eskalen *et al*., (2013). An important note from this study is that there were high infestation levels of PSHB in the Los Angeles Botanical Gardens and Arboretum where reproduction of PSHB was recorded on other hosts and hosts that showed severe signs of dieback. Because PSHB has been demonstrated to attack hosts indiscriminately (Mendel *et al*., 2021) the likelihood of PSHB attack on hosts resistant to fusarium dieback is more likely in areas where population numbers are high. This may explain the reports of PSHB in grapevine in areas with high infestations such as Los Angeles in the USA and George in South Africa.

Although *F. euwallaceae* is here described as non-pathogenic to grapevine, caution should be taken as these were only assessed using few isolates and two cultivars in a single region and under specific production conditions. The effect of environment may play a role in suitability as host in some cases. For example, in Israel and California no attack by PSHB was observed on *Ficus microcarpa* nor was reproduction observed. However, in China, *F. microcarpa* is reported as a reproductive host (Coleman *et al.*, 2019). Furthermore, Ruzzier et al. (2021) pointed out that *Xylosandrus germanus* infestation of a grape vineyard varied throughout the production block. In their assessment, grapevine plants that experienced colonisation were located close to concrete walls which may have provided more shade and moisture retention, thereby providing a possibly favourable environment for colony establishment (Ruzzier *et al*., 2021). The vineyards used the current trial were not irrigated, however effort was made to inoculate plants in different microclimates throughout the vineyard that may experience more shade or moisture retention in the soil. Also, the pathogenicity of the other fungal symbionts, *Graphium euwallaceae* and *Paracremonium pembeum,* towards grapevine is not known. These have proven to be less pathogenic than *F. euwallaceae*, but this has only been determined on *Platanus racemosa* (California sycamore)*, Acer negundo* (Boxelder)*, Persea americana* (Avocado) and *Ricinus communis* (Castor oil plant) (Freeman *et al*., 2016; Lynch *et al*., 2016). Fungal promiscuity of ambrosia beetles has also been recorded by Carrillo *et al*. (2019) in native regions of the PSHB which poses a risk of PSHB obtaining new fungal taxa which could be more virulent to grapevine (Jiang *et al*., 2021).

Management and recommendations

Results from this study indicates that there seems to be no immediate danger posed to grapevines by PSHB and its fungal symbiont *F. euwallaceae* in South Africa. However, introduction and establishment of PSHB into a production site containing reproductive hosts and other susceptible crops grown alongside grapevine such as stone fruit could result in yield losses. These have yet to be assessed for susceptibility to beetle and fungal colonization. South Africa is home to more than one genotype of the beetle and likely also the fungus

(Biermann, pers. comm.). This gives these organisms the capability to evolve virulence and cause future outbreaks on non-usual hosts. New genetic material may also continuously enter the country due to limited support for goods inspections at borders. Continuous monitoring for possible infestations should therefore be conducted to determine if the beetle is present at a site and whether it is able to successfully infest grapevines.

Initial measures that can be taken include limiting movement of green waste and wood to areas near the production site as these are primary local means of spread. Untreated wooden pallets coming into packhouses and around production sites should be inspected for the presence of boring beetles. Early detection of the beetle would allow producers to implement early cultural management methods, such as removal of infested branches, which should be chipped to sizes less than 5 cm and subsequently solarised by covering with plastic sheeting for 4-6 weeks (Eatough Jones and Paine, 2015; Chen *et al.*, 2020). Early removal of infested branches interferes with reproduction cycles of PSHB and thus lowers the number of subsequent colonisation events (Mendel *et al*., 2017). For the establishment of new vineyards, windbreaker trees that do not support beetle or fungal development should be selected. This will reduce spread into production sites as well as reduce chances of increased propagule pressure and possible movement onto grapevines.

There are currently no confirmed methods for the control of established PSHB colonies in trees. Researchers in California and South Africa are in the process of identifying biological control options against PSHB (Stouthamer, pers. comm., Townsend, pers. comm.). While there are no effective chemical treatments currently registered for the control of PSHB in South Africa, the pesticides emamectin benzoate or bifenthrin combined with the fungicide propiconazole show potential as chemical control measures (Mayorquin *et al*., 2018; Eatough Jones and Paine., 2018; Grosman *et al*., 2019). This is currently under investigation in South Africa (Nependa, pers. comm.).

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Table 1: Isolates and sources of *Fusarium euwallaceae* used to inoculate grapevine cultivars.

Isolate	Source	Location	Collector	Date Collected
FR3	*Malus domestica* (Apple)	George	F. Roets	July 2018
FR7	*Vitis vinifera* (Grapevine)	George	F. Roets	August 2019
FR8	*Prunus avium* (Cherry)	George	F. Roets	August 2019

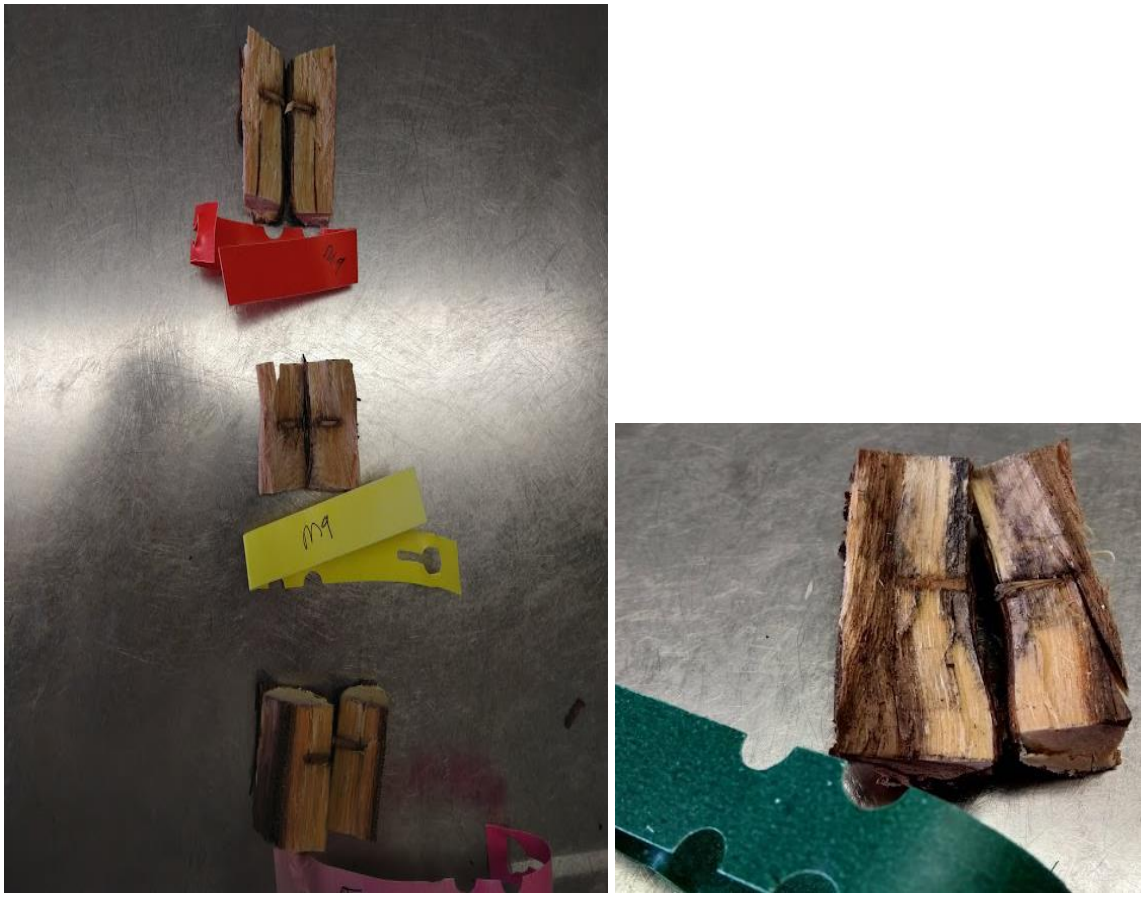

Figure 1: Photo illustration comparing lesion development by *F. euwallaceae* (red = FR3, yellow = FR7 and pink = FR8 tags) compared to control (green = no isolate tag) 1 month post-inoculation.

CHAPTER 3:

Assessment of the pathogenicity of *Fusarium euwallaceae*, symbiont of polyphagous shot hole borer, on deciduous fruit trees in South Africa

Abstract

Euwallacea fornicatus (Coleoptera: Curculionidae: Scolytinae) (polyphagous shot hole borer, PSHB) is a woodboring beetle capable of infesting a wide range of trees in natural, urban, and agricultural settings, and has been reported on important fruit trees in its invaded ranges. Losses occur due to colonization of host sapwood with its fungal symbiont, *Fusarium euwallaceae* (Hypocreales; Nectriaceae), during colony establishment which later leads to Fusarium dieback disease. The potential of fruit tree species in agricultural production settings to develop Fusarium dieback has not been investigated in South Africa. This study set out to determine the host status of apple, nectarine, and three cultivars of plum to *F. euwallaceae* colonisation. Orchards with trees earmarked for removal were selected to be inoculated with multiple isolates of *F. euwallaceae* and lesion development characterised. Differences in factors such as branch diameter, cultivar, host species and isolates in dictating the degree of lesion development was also assessed. It was confirmed, for the first time, that *F. euwallaceae* can colonize and be pathogenic to all host tree species and cultivars tested. The susceptibility of nectarine to *F. euwallaceae* was significantly higher than for plum, but host response did not differ between the two plum cultivars. Branch width and an increase in number of inoculation points per branch did not influence the rate of lesion development. No significant differences in virulence between different *F. euwallaceae* isolates were detected. This study therefore confirms the pathogenicity of *F. euwallaceae* to apple, nectarine, and plum trees in South Africa. Ornamental plum, apple and almond trees were also reported as reproductive hosts for PSHB in South Africa for the first time, albeit from old tree individuals in gardens.

Introduction

The polyphagous shot hole borer (PSHB), *Euwallacea fornicatus* Eichhoff (Coleoptera: Curculionidae: Scolytinae) is an invasive ambrosia beetle in California, Israel, Hawaii, Australia, South Africa, Poland, Italy, Germany and the Netherlands (Eskalen *et al*., 2012; Mendel *et al*., 2012; Paap *et al.,* 2018; Rugman Jones *et al*., 2020; IPPC, 2021; Schuler *et al.,* 2021). The beetle vectors a fungal mutualist, *Fusarium euwallaceae* (Hypocreales; Nectriaceae) that is pathogenic to susceptible hosts and that can cause Fusarium dieback disease (Freeman *et al*., 2013). Its common name refers to the ability of PSHB to colonise a multitude of different host

species. For example, in California (United States), *ca*. 100 hosts have been reported to be susceptible to *F. euwallaceae* and 25% of urban trees are reproductive hosts for PSHB (Leathers, 2015). Presently, over 52 hosts have been reported in Israel (Mendel *et al*., 2012). Since PSHB's initial discovery South Africa in 2017, it has been reported on several economically important trees such as stone fruit, citrus, and nut trees (Paap *et al*., 2018; FABI, 2021). Currently, 130 hosts have been identified in South Africa where 48 have been confirmed to be reproductive hosts (Van Rooyen *et al*., 2021). These include many hosts from native, agricultural, and urban areas. The currently known commercially cultivated fruit-tree species that may host PSHB, or its fungal symbiont includes avocado, apples, citrus, guava, peach, apricot, pecan nut and almond (Eskalen *et al*., 2013; Moreno *et al*., 2017). Of these, avocado (*Persea americana*) is currently the most severely impacted (Eskalen *et al*., 2013; Mendel *et al*., 2012). Previous studies also confirmed the pathogenicity of *F. euwallaceae* to almonds (*Prunus dulcis*) in California (Eskalen *et al*., 2013; Moreno *et al*., 2017).

PSHB can spread rapidly in local areas, mainly by flight and on contaminated wooden products and garden waste (Leathers *et al*., 2015; Faulkner *et al*., 2016; Meurisse *et al*., 2019). Accordingly, it has spread to almost all avocado production regions in Israel in just seven years since its initial detection in 2009 (Mendel *et al*., 2017). While PSHB has not yet caused damage in avocado orchards in South Africa, it has been found in every province except Limpopo, including areas with natural forests and urban landscapes. Over 1000 urban trees in Cape Town have been identified for removal after severe PSHB infestations which has been estimated to cost the city ca. ZAR 3 million (Van Rooyen *et al*., 2021). In addition, *ca*. ZAR 4.5 million will have to be allocated to replace trees that have been removed (Van Rooyen *et al*., 2021). Since the first report of PSHB in South Africa in 2017 (Paap *et al*. 2018), most reports concerning fruit-bearing trees come from individual trees in gardens, which makes it difficult to predict the effects of PSHB infestation in agricultural landscapes.

Damage to hosts is caused by both the physical destruction of xylem tissues due to gallery formation, while PSHB burrows into the xylem of the host, it releases spores of *F. euwallaceae* from specialised structures (mycangia) located below the mandibles (Hulcr & Stelinski, 2017). The fungus colonises the host xylem intercellularly, causing blockages in the vascular tissues and therefore upward translocation of water and minerals. This leads to the plants developing dieback symptoms such as foliar wilting and low fruit set (Eskalen *et al*., 2013). The fungus serves as the primary food source of the beetle (Eskalen *et al*., 2013; Farrell *et al*., 2007). Other fungal mutualists of PSHB include *Graphium euwallaceae* (Microascales; Microascales incertae sedis) and *Paracremonium pembeum* (Hypocreales; Nectriaceae) however these are not pathogenic to the hosts (Freeman *et al*., 2016).

Most severe disease development is a result of PSHB reproduction in hosts. In terms of agriculture, breeding of PSHB has been detected in avocado (USA, Israel, South Africa) and

almond (California) (Eskalen *et al*., 2013; Moreno *et al*., 2017). However, not all hosts are suitable for reproduction (Eskalen *et al*., 2013). The terms 'competent hosts' and 'non-competent' hosts had been used by Lynch *et al*., (2021) to describe hosts able to support beetle reproduction and those that are unable to support beetle reproduction, respectively. 'Killed-competent' hosts are those which die because of beetle and fungal colonisation (Lynch *et al*., 2021). The majority (78%) of genera within the Rosaceae have been found to be 'killed-competent' (Lynch *et* al., 2021). Genera included in Rosaceae are *Prunus, Pyrus* and *Malus*, indicating that various stone fruit and pome fruit species are at risk of significant damage by PSHB. When selecting hosts, PSHB likely does not discriminate between reproductive or non-reproductive hosts (Mendel *et al*., 2021). However, it is attracted to fungal emissions and compounds that it associates with stressed or dying trees (Hulcr and Dunn., 2011; Mendel *et al*., 2017; Li *et al*., 2016; Stouthamer *et al*., 2017). Individual reports from garden settings have provided information of the possible host range of PSHB, however, the susceptibility of these hosts to *F. euwallaceae* in production settings is unknown. Given the risk of trees within the Rosaceae of developing severe dieback, investigations of PSHB and fungal colonization in deciduous fruit trees in agricultural landscapes are quite urgent.

Mendel *et al.,* (2021) proposed three categories to characterise the resistance status of a host to *F. euwallaceae*; (i) those exhibiting resistance to *F. euwallaceae*, (ii) hosts able to slow the development of *F. euwallaceae* and (iii) hosts that are very susceptible to *F. euwallaceae*. Exactly what characterises a host's resistance to the beetle-pathogen complex is unknown, however Lynch *et al*., (2021) studied the phylogenetic relationships between hosts that showed different levels of susceptibility to PSHB attack and disease development. Results indicated that more closely related taxa often showed the same level of susceptibility to PSHB, indicating that the presence or absence of certain familial genes could be conferring resistance (Lynch *et al*., 2021). Interestingly, in the case of avocado, differences in disease severity were also noted between cultivars (Mendel *et al*., 2017). If significant differences between cultivars can be observed, which are genetically much more closely related than distinct species, it is likely that multiple genetic, physiological, and agronomic factors could dictate disease severity. This also suggests that differences in host response of cultivars in other species is possible and should be assessed.

Another factor that may increase severity of Fusarium dieback disease is branch and/or stem diameter of hosts. Mendel *et al*., (2017) noted increased gallery density in avocado branches where the diameter was smaller. Conversely, in box elder trees, it was noted that the wider the trunk, the more galleries were observed in a tree (Mendel *et al*., 2017). It could be hypothesized that the virulence of the fungal symbiont may differ according to branch size, but this aspect has not been investigated. Similarly, multiple PSHB individuals often colonize the same branches on susceptible trees, and it is thought that this cooperative colonization may

help weaken host defences (Mendel *et al.,* 2017). Increased number of colonization points may therefore be correlated to increased ability of the fungus to colonize its host. In other words, fungal colonization may facilitate follow-up colonization, but this has not yet been evaluated.

In South Africa, deciduous fruit orchards covered 54 819 hectares in 2020 (Hortgro, 2020) where apples made up 46% of production areas, pear orchards 25% and stone fruit accounted for 30% of deciduous fruit produced. The sales prices of apples in 2020 were valued at ZAR 10 777.6 million, ZAR 4990.5 million for pears and a ZAR 2671.4 million for stone fruit (Hortgro, 2020). Export of deciduous fruit contributes significantly to the country`s GDP (Gross Domestic Product) and is threatened by biotic and abiotic stresses (Rosenzweig *et al*., 2001).

Introduction of new pests threaten fruit production of deciduous fruit tree species and PSHB is no exception (Rosenzweig *et al*., 2001). To understand the potential impact of PSHB and its fungal symbiont, its effects need to be studied in agricultural production systems.The aim of this study was to assess the threat of the PSHB and its fungal symbiont *F. euwallaceae* in its ability to cause Fusarium dieback on deciduous fruit trees in agricultural production landscapes. In addition, we monitored for colonization of deciduous fruit trees by PSHB in gardens to determine whether the beetles may be able to breed in these species. The objectives were to determine susceptibility of selected fruit trees to *F. euwallaceae* and to determine the effects of factors such as branch diameter, cultivar and number of inoculation points on the susceptibility of hosts to *F. euwallaceae*.

Materials and Methods

Monitoring

The presence of PSHB infestations on possible newly reported hosts in various cities in South Africa were continuously monitored by reacting to reports from the public. When a possible PSHB infestation was reported, samples were taken from entrance holes following protocols set out by the Forestry and Agricultural Biotechnology Institute (FABI, Pretoria, https://www.fabinet.up.ac.za/pdf/PSHB/6-PSHB%20how%20to%20sample20210304.pdf).
These were analysed for the presence of *F. euwallaceae* by surface sterilization of wood using 70% ethanol for 1 min and plating small pieces of wood on petri dishes containing potato dextrose agar (PDA) (Biolab, Midrand, South Africa). These plates were incubated in the dark for 10 days and resulting colonies morphologically assessed for *Fusarium*, purified and the identity confirmed via sequencing of the ITS (internal transcriber spacer) region following PCR (polymerase chain reaction) following Paap *et al*., (2018). To assess whether PSHB was able to breed in tree individuals, tunnels were excavated on the host plants and assessed for the presence of breeding colonies.

Inoculum preparation

Isolates of *F. euwallaceae* from five different source hosts (Table 1) were used in assessment of its pathogenicity towards selected tree species and cultivars. For each tree species/cultivar, three of the five isolates were used (Table 2). These were chosen in accordance to host matching with the species and cultivars evaluated in the current study. For example, the trial testing for pathogenicity of *F. euwallaceae* towards apple in this study, also included an isolate that was originally obtained from an apple host (FR3). Inoculum was prepared using wooden toothpicks (2 mm in diameter) that were autoclaved in potato dextrose agar (PDA) (Biolab, Midrand, South Africa) that was diluted to approximately 1/3 of the recommended concentration. This was done to saturate the toothpicks with media to support fungal growth. The agar-saturated toothpicks were then placed on the surface of petri dishes containing PDA. The selected isolates were inoculated onto petri dishes and allowed to incubate for 14-21 days at 25°C in the dark, until fungal hyphae covered the toothpicks. Similarly, agar-saturated toothpicks were plated onto sterile PDA petri dishes without fungal isolates to serve as controls.

Host taxa and sites

Apple (*Malus domestica* cv. 'Pink Lady'), nectarine (*Prunus persica* var. *nucipersica* 'Alpine') and three cultivars of plum (*Prunus domestica* 'Angelino', 'Fortuner' and 'Sungold') were included in assessments of susceptibility to *F. euwallaceae* in the present study. The apple orchard used for the trial was located on Lourensford estate, Somerset West (-33°35'36"S, 18°.55'53"E) and was still producing fruit. The plum and nectarine orchards were all located at Sandrivier Landgoed in Wellington (-33°, 18.932259E). As with the apple orchard, these trees were still in use for production and were producing fruit as well as showing no signs of disease. Production conditions of the apple and stone fruit orchards used in this trial were ideal and trees were still being tended to and used for fruit harvesting. This included the application of fertilisers and insecticides/fungicides as prescribed and where needed.

Pathogenicity trial protocols

Each host taxon was inoculated with one of three fungal isolates and a control. For the plum cv. 'Sungold' four isolates were used (Table 2). Inoculations were administered by drilling 1 cm deep holes into branches using a 2.5 mm wide drill bit. Branches were selected at random, however selected branches were at least 2 cm thick and only one row of trees in an orchard was used (due to concerns posed by the growers). One branch per tree was inoculated with one of the fungal isolates or the control into five holes drilled in a spiral-like fashion, starting at *ca*. 10 cm from the base of the branch and with 5 cm distance between each hole on the

branch. Toothpicks that were colonised with *F. euwallaceae* or uncolonized controls were placed into each hole, severed at the branch surface, and wounds closed using Parafilm™ to avoid secondary infections by other pathogens. For plum and nectarine trees, eight replicates were performed per isolate and control (n = 32 tree individuals). The total number of plum cv. 'Sungold' trees inoculated was 200 (see later section). Ten individual apple trees were assessed for each of the three isolates and a control (Table 2) (n = 40 individuals).

Inoculations were all initiated during late summer (between 26 January and 17 February 2021) except for apple that was inoculated in autumn (March 2021). The incubation period for each host was determined by date of tree removal or when branch lesions grow close to the tree trunk (Table 3). The incubation time could not be kept consistent as most orchards used in trials were earmarked for removal at various stages. After incubation, branches were harvested by removal at the base of the main stem. The length of the lesion (mm) around each inoculation point was determined by splitting the branch lengthwise and measuring the length of the longest distance of the stained portion of the vascular tissues using digital callipers. Isolations were made from stained portions of vascular tissues to determine whether *F. euwallaceae* was the causal agent for lesion development. Branch sections containing lesions were surface sterilised with 70% ethanol for 1 min after which approximately 5 mm^3 sections from the lesions were plated onto PDA medium in petri dishes. These were incubated in the dark at 25°C for approximately seven to ten days and resulting fungal cultures were morphologically evaluated to confirm identity as *F. euwallaceae*.

Plum cultivar on disease development

Two plum cultivars, Fortuner and Sungold, were inoculated at the same time in the field, had similar incubation periods (five weeks), had individuals with the same number of inoculation points per branch (five), and were inoculated by three of the same fungal isolates (CMW52826, FR8 and CMW53018). Differences in susceptibility to *F. euwallaceae* could therefore be determined between these hosts.

Stone fruit species on disease development

Two species in this study, plum 'Angelino' and nectarine 'Alpine', were inoculated at the same time in the field, had similar incubation periods (five weeks), had individuals with the same number of inoculation points per branch (five), and were inoculated by three of the same fungal isolates (CMW52826, FR8 and CMW53018). This allowed for detection of differences in susceptibility to *F. euwallaceae* between these hosts. Again, the influence of treatment (different isolates and controls), species, and their interaction (as fixed effects) were evaluated

against lesion length data using generalized linear mixed-effect models as described above. This model followed the format: glmer (Lesion length ~ Species + Treatment + Cultivar*Treatment + (1|Tree individual), family = Gamma (link = "log"), data = data).

Fusarium euwallaceae on apple

The effect of treatment with different isolates of *F. euwallaceae* on disease development in apple were assessed nine weeks post inoculation (Table 3). The influence of treatment (different isolates and controls) was evaluated against lesion length data using generalized linear mixed-effect model as described above. This model followed the format: glmer (Lesion length ~ Treatment + (1|Tree individual), family = Gamma (link = "log"), data = data).

Variability in inoculum load on disease development on plum

To determine the effect of increased inoculum load on fungal growth (simulating increased beetle attack), one, three, five and seven holes were drilled into individual branches of plum cv. 'Sungold' as described before. Only one branch per tree was treated with inoculum. Each inoculum load level (one-to-seven) was replicated 10 times for each of four isolates and the control resulting in 200 replicate tree individuals in total. After 5 weeks, lesion lengths were determined. The influence of treatment (different isolates and controls), inoculum load, and their interaction (as fixed effects) were evaluated against lesion length. data using generalized linear mixed-effect models following the formula glmer (Lesion length ~ Treatment + inoculum load + Treatment*Inoculum load + (1|Tree individual), family = Gamma (link = "log"), data = data).

Branch diameter on disease development

To determine the effect of branch diameter on the virulence of *F. euwallaceae,* the diameters of branches that were inoculated with the fungi were measured for apple, nectarine and all plum cultivars. Data for each of these hosts were analysed separately to account for differences in incubation periods. Prior to these analyses, the mean lesion length of all replicates per individual tree was calculated because tree individuals and branch diameters were perfectly correlated (i.e., all inoculations on a tree individual were on the same branch). The influence of treatment (different isolates excluding the controls) and branch diameter were evaluated against lesion length data using a general linear model (Bates and Sarkar 2007) following the formula glm (Mean lesion length ~ Isolate + Branch diameter, family = Gamma (link = "log"), data = data). After analyses, p-values were adjusted for multiple testing using a Bonferroni correction (Perret *et al.,* 2006).

Statistical analysis

Lesion length data were analysed using RStudio® Software (version 3.6.3; Rstudio 153 Team, 2019). The influence of treatment (different isolates and controls), cultivar, and their interaction (as fixed effects) on lesion length data were tested using generalized linear mixed-effect models (Bates *et al*., 2014). Lesion length data best fitted a gamma distribution with log link function (Bolker *et al*., 2009). Tree individual was used as a spatial random variable to improve the model parameters. As these analyses showed no overdispersion of variances compared to the models a Chi^2 statistic and p-value were calculated (Bolker *et al*. 2008). The model followed the format: glmer (Lesion length ~ Cultivar + Treatment + Cultivar*Treatment + (1|Tree individual), family = Gamma (link = "log"), data = data). Significant main effects were separated using conservative Tukey post-hoc tests in the *multcomp* package in R (Hothorn *et al*., 2008). A probability level of 5% was considered significant.

Results

Host status of apple, nectarine and plum

F. euwallaceae was recovered from all inoculated apple, nectarine and plum trees used in the trials, thereby fulfilling Koch's postulates. It is therefore confirmed that *F. euwallaceae* is the causal organism of vascular streaking observed in inoculated plants.

Monitoring

The presence of *F. euwallaceae* was confirmed on samples processed from reports of PSHB attack on apple (*Malus domestica*) and almond (*Prunus dulcis*) trees in Somerset West, Cape Town, South Africa. Breeding colonies of PSHB were also evident in these two hosts. However, in both cases the tree individuals (one each) were very old and seemingly stressed. Younger trees in the vicinity of these showed no signs of PSHB colony formation, even though these were also colonised by *F. euwallaceae*. In addition to these, two trees of *Prunus cerasifera* (purple leaf plum) in gardens in the same town were found to host breeding colonies of PSHB.

Plum cultivar on disease development

The full model had an AIC value of 1921.9 with a deviance of 1899.9, DF = 374. Of the variables included in the model, only treatment had any significant ($p < 0.001$) effect (Table 4). Lesions caused by all isolates were significantly ($p < 0.001$) longer than those in the controls

(Table 4, Figure 1). There was no difference in lesion length between the different isolates ($p < 0.001$) (Table 4, Figure 1).

Stone fruit species on disease development

The overall model had an AIC value of 2042.1 with a deviance of 2022.1, DF = 280. Of the variables included in the model, treatment and host species had significant effects (Table 4). Lesions caused by all isolates were significantly ($p < 0.001$) longer than those in the controls (Table 4, Figure 2). Lesions caused by the Nectarine cv. 'Alpine' were significantly ($p <0.001$) longer than those found in the Plum cv. 'Angelino' (Table 4, Figure 2). There was no difference ($p > 0.05$) in lesion length between the different isolates (Table 4, Figure 2).

Fusarium euwallaceae on apple

The model had an AIC value of 797 with a deviance of 785, DF = 182. Lesions caused by all isolates were significantly ($p < 0.001$) longer than those in the controls (Table 4, Figure 3). There was no difference ($p > 0.05$) in lesion length between the different isolates (Table 4, Figure 3).

Variability in inoculum load on disease development on plum

The complete model had an AIC value of 3516.4 with a deviance of 3472.4, DF = 699. Of the variables included in the model, only treatment had a significant effect (Table 4). Lesions caused by all isolates were significantly ($p < 0.001$) longer than those in the controls (Table 4). There was no difference ($p > 0.05$) in lesion length between the different inoculum loads (Table 4).

Branch diameter on disease development

The model for apple had an AIC value of 247.33 with a deviance of 2.9277, DF = 27, the model for the plum cv. 'Angelino' had an AIC value of 206.41 with a deviance of 4.513, DF = 27, and the model for nectarine had an AIC value of 247.33 with a deviance of 2.9277, DF = 27. In all cases only treatment had a significant effect (Table 4). Lesions caused by all isolates were significantly ($p < 0.001$) longer than those in the controls, but similar to each other (Table 4). There was no difference (> 0.05) in lesion length between the different branch diameters in any tested species or cultivar (Table 4).

Discussion

This study investigated the pathogenicity of *F. euwallaceae* on selected deciduous fruit trees in agricultural production landscapes in the Western Cape province of South Africa. For all hosts, lesions caused after inoculation by *F. euwallacea* were significantly longer than that of controls, and this fungus was successfully recovered from all field trials. The pathogenicity of *F. euwallaceae* to plum, nectarine, and apple trees is therefore confirmed. *Prunus* spp. in general seem to be susceptible to *F. euwallacea* as multiple species are known as hosts of the fungus in South Africa and abroad (Eskalen *et al*., 2013; https://www.fabinet.up.ac.za/pshb). It also seems as if numerous species may act as breeding hosts for PSHB as confirmed here for *P. cerasifera* and *P. dulcis*. There is therefore elevated risk of severe impact caused by this invasion on stone fruit production in South Africa. Similarly, production of apple may also be at risk as the fungus is pathogenic to it and PSHB may be able to breed in at least stressed, older trees. These results, and those in other studies, seemingly point towards a general elevated level of susceptibility of tree species in the Rosaceae to *F. euwallaceae* and PSHB (Lynch *et al*., 2021). The theoretical approach to identify reproductive hosts of PSHB developed by Lynch *et al*., (2021) therefore provides a useful initial screening tool for identifying candidate tree species that should be investigated for susceptibility to Fusarium dieback.

No differences in lesion development between any of the isolates used and on any host were found. This, despite the isolates originating from localities and hosts that differ substantially in distance and identity, respectively. This is likely testament to a recent invasion by only a limited number of PSHB individuals and therefore also likely a nearly clonal population of *F. euwallaceae* present in the country. This also means that there is currently little chance of the beetle or the fungus evolving to become greater risks than currently estimated. However, with introductions of new genetic variants into existing populations chances of development of individuals with enhanced virulence is possible (Schuler *et al*., 2021). Therefore, additional introductions of PSHB and its fungal symbionts into the country should be avoided.

The number of inoculation points and branch size had no influence on fungal growth rates indicating that the fungus growth is constant irrespective of host size (or age) or the level of attack by PSHB on the tree, although increased excavation by PSHB and inoculation of *F. euwallaceae* will certainly increase disease expression. Therefore, for the purposes of determining susceptibility of trees to *F. euwallaceae,* a single inoculation point to a branch or stem of multiple individuals would suffice. However, multiple inoculation points may be needed when characterising dieback symptoms in branches of hosts.

Comparisons of variation in lesion lengths between the plum cvs. ‘Sungold’ and ‘Fortuner’ revealed no difference. This contrasts with what has been found between different avocado cultivars (Eatough Jones & Paine, 2017). This may point towards a general pattern of cultivar

susceptibility towards *F. euwallaceae*, only a limited number of cultivars were assessed. Finding differences in cultivar susceptibility is important, as this can form the basis of any resistance breeding programme should PSHB become a significant pest in production areas. Differences in susceptibility between different host species to *F. euwallaceae* was however apparent here, indicating that all stone fruit taxa should be evaluated to have a global idea of possible risk. At present, 35 nectarine, 25 plum and prunes and 13 apples, represented in Hortgro (2020), statistics are produced in South Africa. The most popular cultivars of nectarine are 'Arctic Wolf', 'Giant Pearl', 'Arctic Star', 'Polar Light', 'Arctic Runner', 'Primrose' and 'Honey Royale' (Hortgro, 2020). Plum and prune cultivars most often produced include 'Angelino', 'Laetitia', 'Ruby Sun', 'African Delight', 'Fortuner', 'Ruby Star' and 'Sungold' (Hortgro, 2020). The apple cultivars that contribute the most to production include 'Golden Delicious', 'Royal Gala', 'Granny Smith', 'Pink Lady', 'Starking', 'Fuji' and 'Joya' (Hortgro, 2020). These cultivars should be screened forlevels of susceptibility, particularly if there are cultivars able to tolerate certain levels of beetle infestation without economically significant yield loss. However, many other cultivars may not be as commonly utilised across South Africa because of unfavourable agronomic characters, lack of consumer interest or proneness to various plant diseases. Although these may be useful when screening for resistance traits in endeavours for cultivar improvement.

At present, management practices of PSHB in orchards are limited to early detection and timely application of cultural control methods (Eatough Jones & Paine, 2015; Mendel *et al*., 2017; Chen *et al*., 2020). Removal of infested branches and reproductive host trees that surround orchards will be necessary, even if PSHB cannot breed in the fruit trees. Reproductive host trees near orchards will act as reservoirs of infective beetles that can continuously inoculate the fungus into orchard trees. After removal of infested plant material, these should be treated by chipping to sizes < 5 cm and subsequent solarization under plastic sheeting for 4-6 weeks (Eatough Jones & Paine, 2018; Mendel *et al*., 2017; Chen *et al*., 2020).

An important field observation in the nectarine orchard was that the lesions caused by *F. euwallaceae* grew very fast and ended up close to the point of branching from the main stem before the branch itself showed any exterior symptoms of dieback. This means that, if producers choose to prune infested branches only after signs of disease is seen on the leaves and other outer parts of the tree, the infection has likely spread to the trunk and may be difficult to control. On both *Prunus* and apples these can easily be detected as areas where frass is observed and where gum exudates accumulate from small holes. Although pathogenicity of *F. euwallaceae* can be useful as an initial screening tool for potential host susceptibility to developing Fusarium dieback, it does not definitively indicate suitability for PSHB colonisation. That being said, continuous gallery formation by PSHB throughout hosts are required for *F. euwallaceae* infection to cause xylem vessel occlusion as the fungus is not able to sufficiently spread to adjacent tracheids but rather longitudinally throughout the tree's vascular bundle. It is

therefore recommended that branches be pruned if more than one beetle entry hole is observed as this can be a good indication of colony establishment.

Investigations of possible biological and chemical control methods is underway (Stouthamer., pers. comm.; Eatough Jones & Paine, 2018; Mayorquin *et al.,* 2018; Grosman, Eskalen & Brownie, 2019), but it is expected that repeated application of these would be necessary, resulting in further production expenses. It also must be noted that all orchards used in the present study was still subject to chemical treatments including the use of systemic fungicides, that did not seem to affect the ability of *F. euwallaceae* to rapidly colonise the branches. Chemical treatments should therefore undergo scrutiny to show their efficacy.

Investigation of physiological traits associated with reduced disease development would prove useful in refining cultural practices as well as to drive future fruit tree breeding initiatives for resistant varieties. Effector proteins, genes involved in the virulence of *F. euwallaceae,* have been identified by Tyler *et al*. (2019). Similar efforts by Pérez-Torres *et al*. (2021) characterised the infection process in avocado by *Fusarium kuroshium.* Together these data can be used to develop resistant tree varieties. For example, of the 57 differentially expressed genes detected in the latter study, genes associated with alcohol metabolism were the most upregulated, indicating an enzyme group important for secondary metabolite detoxification in hosts (Pérez-Torres *et al*., 2021). Identification of host genes involved in resistance to detoxification efforts of *F. euwallaceae* can lead to better management of the disease in future.

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Table 1: Isolates used in field trials, with their collection details

.

Isolate name	Source	Province	Collector	Date isolated
FR3	*Malus domesitca* (Apple)	George, Western Cape	Francois Roets	July 2018
FR7	*Vitis vinifera* (Grapevine)	George, Western Cape	Francois Roets	August 2019
FR8	*Prunus avium* (Cherry)	George, Western Cape	Francois Roets	August 2019
CMW53018	*Prunus nigra* (Black plum)	Hurlingham, Gauteng	Trudy Paap	May 2018
CMW52826	*Prunus Africana* (African cherry)	George, Western Cape	Willem Z. De Beer	June 2018

Table 2: Isolates used for specific trials on the various host taxa

Host species	Isolate
Malus domestica (Apple) cv. 'Pink Lady'	FR3
	FR7
	FR8
	FR8
Prunus domestica (Plum) cv. 'Angelino'	CMW52826
	CMW53018
	FR8
Prunus domestica (Plum) cv. 'Fortuner'	CMW52826
	CMW53018
	FR3
Prunus domestica (Plum) cv. 'Sungold'	FR8
	CMW52826
	CMW53018
	FR8
Prunus persica var. nucipepersica (Nectarine) cv. 'Alpine'	CMW52826
	CMW53018

Table 3: The incubation period used of *F. euwallacea* on different tree species and cultivars.

Tree species	**Cultivar**	**Incubation period (weeks)**
Malus domestica (Apple)	Pink Lady	9
Prunus domestica (Plum)	Angelino	16
Prunus domestica (Plum)	Fortuner	5
Prunus domestica (Plum)	Sungold	5
Prunus persica var. *nucipepersica* (Nectarine)	Alpine	16

Table 4: The effect of selected variables on the development of *F. euwallaceae* lesions in different fruit tree species and cultivars.

Variable	df	X^2	P-value	Post Hoc
Plum cutivar 'Fortuner' vs. Plum cultivar 'Sungold				
Cultivar	4	2.9048	0.5739	n.a.
Treatment	7	99.942	<0.001	Figure 1
Cultivar*treatment	3	0.412	0.9378	n.a.
Nectarine cv. 'Alpine' vs. Plum cv. Angelino				
Species	4	28.937	<0.001	Figure 2
Treatment	6	74.99	<0.001	Figure 2
Species*treatment	3	6.7588	0.08	n.a.
Apple susceptibility				
Treatment	3	50.343	<0.001	Figure 3
One vs. Three vs. Five vs. Seven Inoculations				
Inoculum load	15	13.254	0.5827	n.a
Treatment	16	243.14	<0.001	Figure 1
Inoculum load*Treatments	3	4.355	0.2256	n.a
Branch diameter				
Apple treatment	30		<0.001	Figure 3
Branch diameter	28		0.5727	n.a
Plum cv. 'Fortuner' treatment	30		<0.001	Figure 1
Branch diameter	28			n.a
Plum cv 'Angelino' treatment	30		<0.001	Figure 2
Branch diameter	28		0.8869	n.a
Nectarine cv. 'Alpine' treatment	30		<0.001	Figure 2
Branch diameter	28		0.5727	n.a

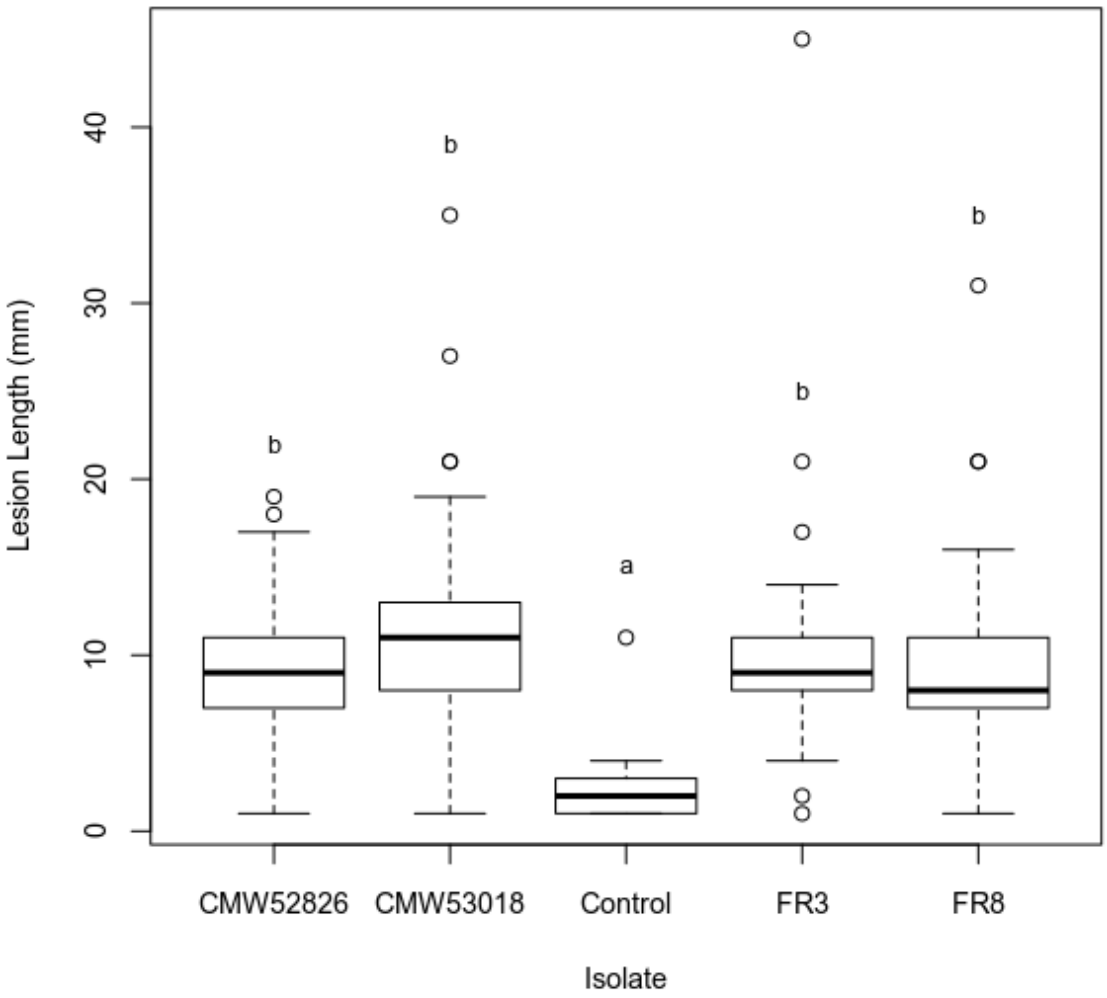

Figure 1: Lesion lengths of different isolates of *Fusarium euwallaceae* in two plum cultivars (Plum cvs. 'Sungold' and 'Fortuner'). Data for the two host taxa were combined as there were no significant differences between them. Boxes indicate 25–75% data range, whiskers indicate 1.5 times the interquartile range and dots represent outliers.

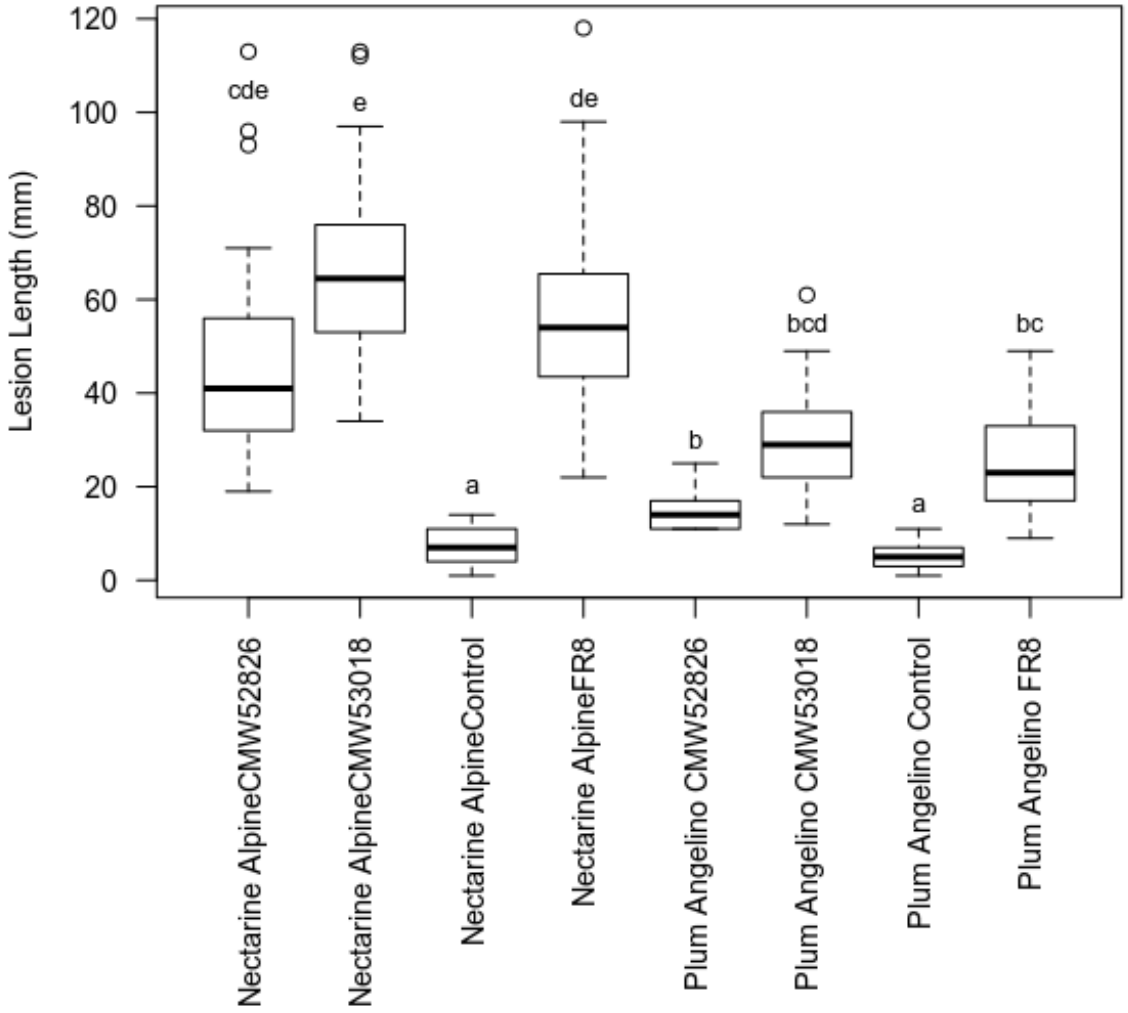

Figure 2: Lesion lengths of different isolates of *Fusarium euwallaceae* measured in plum cv. 'Angelino' and Nectarine cv. 'Alpine'. Boxes indicate 25–75% data range, whiskers indicate 1.5 times the interquartile range and dots represent outliers.

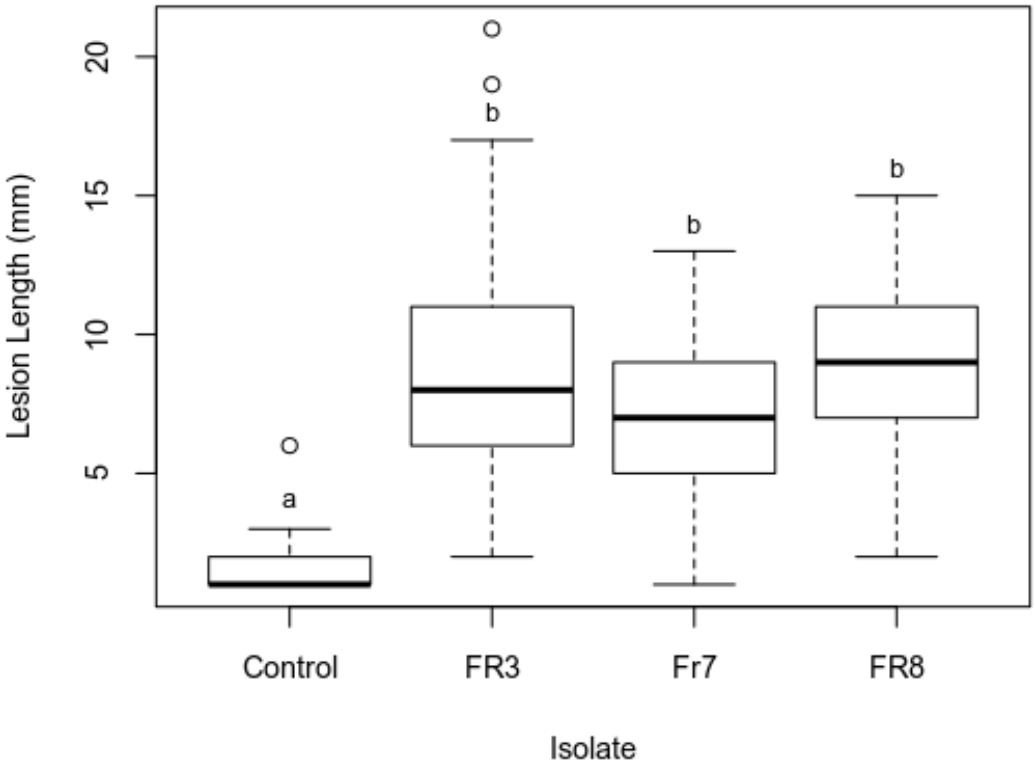

Figure 3: Lesion lengths from different isolates of *Fusarium euwallaceae* treatments in apple cv. 'Pink lady'. Boxes indicate 25–75% data range, whiskers indicate 1.5 times the interquartile range and dots represent outliers.

CHAPTER 4:

Rapid, cost-effective detection of *Fusarium euwallaceae* from woody tissues

Abstract

The polyphagous shot hole borer, *Euwallacea fornicatus* Eichhoff (Coleoptera: Curculionidae: Scolytinae) (polyphagous shot hole borer, PSHB) is an invasive ambrosia beetle that infests a wide variety of trees. Native to Asia, PSHB has now spread to many countries around the world and threatens hundreds of tree species in native, urban and agricultural areas. To establish within a host, PSHB relies on mutualistic fungi, most notably the plant pathogen *Fusarium euwallaceae* (Hypocreales; Nectriaceae). *Fusarium euwallaceae* can cause Fusarium dieback disease which may lead to the death of highly susceptible hosts. Key to the mitigation of the spread and control of PSHB is rapid detection. This is hampered by many factors including its cryptic ecology and biology and very wide host range. Also, detection and identification are hampered due to the need for specialised identification techniques for both the beetle and the fungus. Current identification relies on DNA extraction and sequencing of the beetle and/or *F. euwallacea* that is expensive and time consuming. Especially in the case of *F. euwallaceae*, extra time for identification is needed as a viable colony needs to be established first from infected material before DNA sequencing procedures. Because PSHB has a strong association with its fungal symbiont, and the fungus can infect trees without PSHB establishing viable colonies, a molecular tool that can rapidly detect specifically *F. euwallaceae* would be beneficial in monitoring efforts. A protocol that uses species-specific primers to amplify DNA of *F. euwallaceae* directly from infected wood of various tree species in both artificial and natural conditions was developed. The protocol was able to detect this fungus within minimal woody material (*ca.* 0.015 g) and even from degraded/dry samples of many, but not all hosts. Even though the protocol does not work for all host species, these included some of the most important agricultural (e.g., avocado) and urban trees (e.g., London Plane and Boxelder) that are severely impacted by PSHB. Additionally, direct PCR using lysate prepared from isolate mycelia recovered from infected xylem provided a reliable secondary measure where amplification of target gene from diseased wood was not possible in certain hosts. A PCR protocol that allows for the identification of *F. euwallaceae* directly from diseased xylem tissues of many hosts, without the need for prior DNA extraction or fungal cultivation, drastically reducing time and costs for monitoring and expanding monitoring capacity, was established.

Introduction

The polyphagous shot hole borer (PSHB), *Euwallacea fornicatus* (Coleoptera: Curculionidae: Scolytinae) is a woodboring ambrosia beetle native to Asia, but it has now successfully invaded Hawaii and California in the United States of America, Israel, South Africa and Australia (Eskalen *et al*., 2012; Mendel *et al*., 2012; Paap *et al*., 2018; Rugman-Jones *et al*., 2020; IPPC, 2021). In South Africa, PSHB had spread to every province except Limpopo representing one of the largest outbreaks of the beetle world-wide (Van Rooyen *et al*., 2021). One reason for this invasive success is ascribed to its wide host range. PSHB has been recorded from over 207 tree and woody plant species in the USA and 130 hosts in South Africa (Leathers, 2015; Van Rooyen *et al*., 2021). It can cause the death of healthy highly susceptible host tree species including important species in agricultural, urban and natural settings. The impacts of the invasion can therefore be severe. For example, over 1000 trees in urbanized Cape Town, South Africa have been earmarked for removal within only two years since its initial detection, which will cost the city *ca*. ZAR 3 million. A further ZAR 4.5 million would be required to replace these trees (Van Rooyen *et al*., 2021). Impacts on agriculture includes costly infestations of avocado orchards in Israel and in California (Eskalen *et al*., 2013; Mendel *et al*., 2017). Impacts in native systems are currently under investigation in South Africa (Van Rooyen *et al*. 2021).

PSHB carries spores of is fungal symbionts, notably *Fusarium euwallaceae,* (Hypocreales; Nectriaceae) within oral mycangia (Hulcr *et al*., 2007; Kasson *et al*., 2013). During gallery excavation in host trees, the fungal spores of *F. euwallaceae* are released and subsequently colonise the xylem tissues (Hulcr *et al*., 2007). The fungus serves as the main food source for the mature beetles and their larvae (Freeman *et al*., 2016; Lynch *et al*., 2016). During fungal colonization, xylem vessels are blocked that prevents the movement of nutrients and water in the three, leading to Fusarium dieback disease and death of highly susceptible hosts (Mendel *et al*., 2017). However, not all hosts are suitable for reproduction, and on many the establishment of colonies of PSHB fail even though *F. euwallaceae* may still colonise the host. There is therefore differentiation between hosts in which 1) the fungus can establish, and beetle can reproduce, 2) those in which the fungus can establish but the beetles cannot reproduce and 3) those that are not suitable for either the beetle or the fungus (Mendel *et al*., 2021). Many hosts from the first category are highly susceptible to these infestations and suffer from Fusarium dieback disease and death, while those in the secondary category are unlikely to die even though Fusarium dieback symptoms may occur (Mendel *et al*., 2012; Eskalen *et al.*, 2012,2013).

Rapid identification of infestations by PSHB is notoriously difficult, but imperative for mitigation efforts. PSHB forms part of the *E. fornicatus* species complex (Coleoptera: Curculionidae: Scolytinae) which also includes three other ambrosia beetles that are

morphologically nearly indistinguishable from each other and *E. fornicatus*: *Euwallacea perbrevis*, *Euwallacea fornicatior* and *Euwallacea kuroshio* (Gomez *et al*., 2018). Molecular techniques are therefore needed for correct identification of the beetle even in its invaded ranges which necessitates fresh samples for DNA extractions (Gomez *et al*., 2018). Also, in many host trees only the fungus can be found without the establishment of beetle colonies. Therefore, monitoring and host identification procedures often rely on the detection of the *F. euwallaceae* mutualist in affected trees (e.g., Van Rooyen *et al*, 2021). This is possible as, even though the different *Fusarium* species that associate with the different *Euwallacea* beetle species are promiscuous in the native region (Carrillo *et al*., 2019; Jiang *et al*., 2021; Lynn *et al*., 2021), this has not been detected in the invaded regions (Kasson *et al*., 2013; O'Donnell *et al*., 2015). Correct detection and identification of *F. euwallaceae* from woody samples are currently hampered by the need to first isolate the pathogen onto a medium that supports microbial growth, growing the fungus, extracting its DNA, performing a PCR using a microbial barcode gene marker and sequencing the PCR product to analyse and compare the target sequence to a data base of organisms that could provide subsequent identification. These protocols are time consuming and expensive, and especially problematic in countries with minimal resources directed toward pest monitoring. There is therefore an urgent need to develop a rapid and cost-effective technique to monitor the spread of PSHB both locally and internationally and to identify hosts susceptible to developing Fusarium dieback.

Recent developments for diagnosis of *F. euwallaceae* using PCR multiplex reactions provides visual diagnosis using gel electrophoresis without the need for DNA sequencing (Short *et al*., 2017; Carrillo *et al*., 2020). With the increase in availability of numerous fungal genomes and transcriptomes in public databases it has become more readily possible to identify novel primer pairs that target genes specific to certain species *in silico*. For example, Short *et al*. (2017) used a comparative genomics approach to identify species-specific protein-coding regions, and to develop species-specific primer pairs for detection and identification of *F. euwallaceae* and many of its closely related species form the Ambrosia Fusarium Clade (AFC) (Kasson *et al*., 2013; O'Donnell *et al*., 2015; Freeman *et al*., 2016). The primer sets were able to correctly identify these closely related fungal taxa from genomic DNA extracted from beetle heads, however the primer pair specific to *F. euwallaceae* was not tested directly on *E. fornicatus* beetle heads (Short *et al*., 2017). Vázquez-Rosas-Landa *et al*. (2021) also used a comparative genomic approach to develop accurate diagnostic systems for various Ascomycete fungi, one of which included a diagnostic PCR marker for *F. kuroshium* by amplification of a specific DNA fragment from DNA extracted directly from symptomatic plant tissues (without needing to culture the fungus). Using PCR to identify *F. euwallaceae* within plant material without prior DNA purification and using species-specific primers has not yet been attempted.

Direct PCR of fungal samples in plant material is not always possible as polyphenols tend to bind to DNA when oxidised (Peterson *et al*., 1997), which can inhibit DNA *Taq* polymerase activity during PCR. Few protocols have been published for direct PCR of fungi in diseased woody tissue, particularly without any form of DNA purification (Lin and Walker., 1997; Kim *et al*., 1995; Langrell *et al*., 2005). Many approaches use modified extraction protocols that contain a more simplified reagent list but may still require expensive equipment to macerate hard tissues (in the case of seeds and wood) and contain hazardous reagents that still require time and labour to prepare (Steiner *et al*., 1995; Lin and Walker *et al*., 1997; Langrell., 2005). High performance PCR reagents such as *Taq* polymerase are also often required to obtain detectable PCR product (AlShahni *et al*. 2009; Cascella *et al*. 2015). Interestingly, the reagents used for simplified or crude DNA extraction protocols vary considerably, except generally for the addition of polyvinylpyrrolidone (PVP) nucleic acid preparations when isolating DNA from plant samples. (Xin *et al*., 2003). PVP has also previously been included directly in PCR mixtures (Dobha *et al*., 2014). PVP reduces polyphenic binding to DNA by removing polyphenol contaminants and preventing oxidation (Loomis *et al*., 1974). In addition, Ben-Amar *et al*., (2017) established a universal direct PCR amplification system where only TE-buffer is used to prepare samples before adding to PCR reagent mixture. TE-buffer is incorporated into DNA extraction protocols due to its role in solubilizing DNA and simultaneously protecting it from degradation (Panda *et al*., 2019). Ben-Amar *et al*., (2017) overcame inhibition of *Taq* polymerase by using a small amount of DNA lysate and a highly sensitive *Taq* enzyme (GoTaq® DNA polymerase (Promega), however this approach requires specific conditions such as working on ice trays or cooling blocks to avoid premature activation of *Taq* polymerase. It would be ideal to also overcome the need for working on cooled surfaces, especially when only a visible PCR product is required for organism detection and no subsequent sequence analysis is needed.

The purpose of this study was to use the *F. euwallaceae* species-specific primer sequences developed by Short *et al*. (2017) and optimise a protocol for its detection with direct PCR from symptomatic plant tissues using readily available reagents as part of a rapid and cost-effective identification protocol. By reducing the number of steps to PCR and subsequent product analysis, such a technique would reduce the chance of contamination, and thus, misidentification at each step. As growers and tree owners are often apprehensive about having trees destructively sampled for the purpose of disease diagnosis, a method that only requires a small sample from beetle entry holes or shavings from symptomatic wood was tested. Also, colonised wood samples sent for diagnostics are often received in poor/dry condition that prevents isolation of fungal causal agents. The rapid technique developed here was therefore tested on samples that have been dried before validation.

Materials and Methods

Inoculation of plant material with Fusarium euwallaceae

Avocado branches were inoculated with *F. euwallaceae* isolate CMW53018 (Isolated from Black Plum [*Prunus nigra*] by Paap, T. in Hurlingham, Gauteng province, South Africa). Inoculum was prepared using toothpicks that were autoclaved in potato dextrose agar (PDA) (Biolab, Midrand, South Africa), which was prepared using 1/3 of the concentration recommended by the manufacturer to saturate the toothpicks and to encourage fungal colonisation (Chapter 2). Saturated toothpicks were placed onto petri dishes containing PDA which were hereafter inoculated with *F. euwallaceae* and incubated for two weeks. Following field inoculation techniques described in previous chapters, 2.5 mm diameter holes were drilled into avocado branches (10 holes per branch) in Stellenbosch, South Africa and *F. euwallaceae*-colonised toothpicks were inserted and broken off at the surface-level of the bark. The inoculation points were sealed using Parafilm™ and allowed to incubate for 11 days. Resulting fungal lesions in sapwood were exposed by removing the outer bark and lesion tissues were removed using a sterile scalpel. Approximately 5 mm^3 of each lesion sample, separated into four equal parts, was plated onto fresh PDA plates and monitored for the growth of *F. euwallaceae* to confirm the identity of the lesion-causing organism following procedures outlined in previous chapters.

Detection in inoculated avocado

Approximately 0.015 g of fresh lesion tissue (discoloured tissues) and 150 µl TE-buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) was deposited into 1.5 ml Eppendorf tubes and macerated for 30 s using a plastic pestle, mixed using a vortex mixer for 15 s, and incubated at 60°C for 10 min, after which the tissue was macerated for another 30 s and vortexed again for 3 s. Hereafter the tubes were centrifuged at 5000 g for 5 s, while 1 µl of lysate was used for PCR. To test sensitivity of the PCR towards partially infected tissues, samples extracted from the lesion front found in wood that contained *ca*. 50% colonised tissue and 50% healthy plant tissue were also prepared. Healthy avocado xylem tissues were prepared in the same way and served as negative controls for PCRs. These experiments were repeated four times, each time using material from a different lesion/inoculation point or uncolonized area on a branch. Genomic DNA that was extracted directly from *F. euwallaceae* cultures using a Qiagen DNA plant genomic extraction kit (Qiagen, USA) served as the positive control. For PCR, reactions that contained ddH_2O instead of DNA or lysate served as a second negative control.

To prevent possible inhibitory effects of plant and fungal phenolic compounds on DNA *Taq* polymerase activity, polyvinylpyrrolidone (PVP) (molecular weight 40 000; Sigma-Aldrich) was

added to the PCR mixture. To determine the optimum concentration of PVP to add to the PCR mixture, volumes of 0, 1, 2, 3, 4 and 5 µl of a 2% w/v PVP solution were added, and the volume of sterile distilled water added was adjusted accordingly. Mixtures of each tested volume of PVP were replicated four times to ensure repeatability. PCR mixtures contained 12.5 µl Ampliqon 2X MasterMix Red, 10 µl sterile distilled water, 1 µl 10 mM of each of the forward and reverse primer, and 1 µl fungal genomic DNA or lysate. PCRs were conducted in 25.5 µl reaction mixtures using a Labnet MµltiGene OPTIMAX thermal cycler. The thermocycler was set to the following conditions: 95°C for 2 min, followed by 38 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 60 s. The *F. euwallaceae* species-specific forward and reverse primers used were obtained from the study of Short *et al*., (2017). These amplify a 260 base pair fragment of a fungal transcription factor regulatory middle homology region (GenBank ID: KT835019). Extensive testing of these primers in this previous study showed that they are sufficient in discerning between *F. euwallaceae* and its closely related members in the ambrosia fusarium clade (Short *et al*., 2017).

After PCR, 12 µl reaction mixture was loaded onto a 1.5% (wt/vol) agarose gel containing 8 µl SYBR Safe DNA gel stain (Thermo Fisher Scientific) along with 8 µl PCRBio IV DNA ladder (PCR Biosystems Ltd.) and subjected to electrophoresis at 95 V for 1 hour and 20 min in 1X TAE (Tris-acetate-EDTA) buffer and visualised using a DarkReader transilluminator (Clare Chemical Research). Fragment sizes of 260 base pairs in length that were visible on the agarose gel were considered a positive result for the presence of *F. euwallaceae* in host tissues.

Detection in field-collected host material

The ability to detect *F. euwallaceae* in hosts that were naturally colonised by *E. fornicatus* in the field was tested by collecting fungus-stained vascular tissues surrounding suspected PSHB entrance holes on 11 host species including avocado, London plane (*Platanus x acerifolia*), English oak (*Quercus robur*), Lombardy poplar (*Populus nigra*), black wattle (*Acacia mearnsii*), boxelder (*Acer negundo*), bugweed (*Solanum mauritianum*), American sweetgum (*Liquidambar styraciflua*), cork oak (*Quercus suber*), coastal coral tree (*Erythrina caffra*) and pin oak (*Quercus palustris*) in Somerset West, South Africa. From each infested host, bark was removed from an area of 3 x 3 cm surrounding a suspected PSHB entrance hole. The sapwood containing the lesion (presumably caused by *F. euwallaceae*) and surrounding uncolonized material was excised to a depth of 5 mm using a sterile chisel and placed in separate sterile petri dishes and stored at 4°C until processing in the laboratory (the day after collection).

In the laboratory, samples were surface sterilised and small pieces of fungus-stained woody material was excised and plated onto PDA media as described before (Chapter 2).

These plates were incubated at 25°C in the dark and monitored for the presence of *F. euwallaceae* colonies. The remainder of each sample was split into two. One half was used for PCR of freshly collected plant material and the other half was left to dry out in a laminar flow cabinet for two days at ambient temperatures (*ca*. 23°C) before processing for PCR. From each half of the sample, plant material was collected for PCR as described above from both the stained tissues and the surrounding non-stained tissues. Therefore, for each collected sample four sub-samples were assessed using the developed PCR technique, including fresh sample from fungus-colonised material, fresh sample from fungus uncolonized material, dried sample from fungus-colonised material and dried sample from uncolonized plant material. Material of both fresh and dried samples containing only 50% stained wood (lesion) were also assessed. PCR conditions were based on the results of the optimal PCR reagent mixture as identified when tested on avocado (as above) and fragment visualisation followed procedures outlined above. Lysate prepared from avocado lesions served as positive controls and lysate prepared from healthy tissues from each host served as negative controls.

Detection in host material collected using a drill

Using a 2.5 mm drill bit attached to a battery-operated drill, wooden dust samples of fungus-stained wood were collected into sterile 1 mL Eppendorf tubes in the field. The drill bit was sterilised before collection of each sample. Samples were taken *ca*. 5 mm directly below PSHB entrance holes where fungal staining is expected but avoiding the entrance hole itself. Only dust originating from the sapwood was used, therefore, dust originating from the bark itself was disregarded. This was done by drilling through the bark until there was a distinct change in the colour of the dust emerging from the hole. At this point the drill bit was surface sterilised and subsequently used to collect *ca*. 1.5 g of fungus-stained sapwood. Samples were stored at ambient temperatures until processing in the laboratory following methods described above. Dark or stained saw dust was separated from lighter saw dust and only the darker saw dust was prepared for the PCR to increase total concentration of diseased tissue. In total, five samples per tree species (same tree species as above) were collected and processed in this manner (each one from a different tree individual when possible). Lysate prepared from avocado lesions served as a positive control and that from uncolonized tissues collected from each host species served as negative controls.

To verify the identity of the lesion-causing organisms collected in this study as *F. euwallaceae*, lysate was also prepared from only TE buffer and fungal hyphae. The tip of a scalpel was used to pick up only a few strands of hyphae from fungal colonies of isolates CMW53019, CMW52826, FR3 and FR8 (previously confirmed as *F. euwallaceae*, Chapters 2 and 3) and all fungal cultures collected from lesions of hosts in the field in the present study and

placed in 50 µl TE buffer. The hyphae were macerated, mixed, and incubated at 60°C for 10 min, after which tubes were centrifuged at 5000 g for 5 s and 1 µl of lysate was used for PCR as described before.

Results

Detection in inoculated avocado

PCR products of the expected size were not produced during the PCR of lysate prepared from fresh stained avocado host tissues with the addition of either 0 or 1 µl PVP, aside from the positive control (Table 1, Figure 1). With the addition of 2 µl PVP to lysate, visible bands were produced. The addition of 3 µl PVP showed the highest sensitivity as this reaction was the only one to amplify the healthy and diseased tissue mixture (50% healthy tissue – 50% diseased tissue lysate). PCR reactions with the addition of either 4 or 5 µl PVP showed signs of reaction inhibition. These results were identical for all experimental replicates.

Detection from field-collected host material

As the PCR reaction mixture containing 3 µl PVP showed the strongest amplification of *F. euwallaceae*-infected avocado woody tissues, this same reaction mixture was used in subsequent testing of detectability of this fungal DNA from other host species. There was strong variability in the detectability of *F. euwallaceae* between the different hosts and the different treatments of the samples (fresh or dry, Table 2). *Fusarium euwallaceae* was detectable from lysate prepared from both fresh and dried lesion material of all samples from avocado, black wattle and boxelder (Table 2). However, in other hosts, positive amplification was only obtained for some samples. Detectability of *F. euwallaceae* was either improved or decreased after drying of the woody samples, depending on the specific host species (Table 2). For example, increased detectability was evident for samples collected from pin oak and bugweed but decreased for London plane. Generally, when combining the results obtained from a wet sample and its dried equivalent, positive amplification results were obtained for all samples collected in this study except for all five samples from English oak, one sample from London plane and three from the coastal coral trees (Table 2). Halving the amount of lesion material in the sample lysate generally reduced detectability in all host species. *Fusarium* cultures were obtained from all samples except one of the samples obtained from London plane. This sample did however result in successful PCR amplification indicating the presence of *F. euwallaceae*. All fungal cultures obtained in this study tested positive for direct PCR amplification using lysate prepared from fungal hyphae indicating the presence of living *F. euwallacea* in all sampled material.

Detection in host material collected using a drill

Lysate obtained from lesion sawdust from English oak did not produce any detectable PCR product (Table 3). In general, detectability of *F. euwallaceae* was reduced when using saw dust collected using a drill bit rather than the collection of infected host material from created wounds. Only one faint band was observed for five samples collected from each of coastal coral, cork oak and pin oak. Three samples from London plane produced bands. American sweet gum and bugweed only had two samples that produced detectable product. Three samples from Lombardy poplar produced bands, four from black wattle and all five from boxelder and avocado.

Discussion

The purpose of this study was to develop a rapid diagnostic tool for Fusarium dieback disease caused by *F. euwallaceae* by using a species-specific PCR marker and a rapid nucleic acid preparation method from woody tissues. It was shown that amplification of a *F. euwallaceae*-specific molecular marker was possible without prior cultivation of the fungus or extraction and purification of DNA from infected woody plant material. Amplification was possible from 10 out of the 11 host species tested indicating a high level of transferability of this method between different host species. In addition, detectability was possible, or even increased after drying of samples. Excluding the five samples from English oak that could not produce detectible PCR products, PCR amplification was possible for 46 of the 50 (92%) of samples collected using the open wound collection method, when combining results from both the dried and the fresh preparations. This represents a very high level of repeatability of detection of *F. euwallacea* in a variety of infected woody material. This not only bodes well for rapid and cost-effective monitoring of *F. euwallaceae* (and, by proxy, also the polyphagous shot hole borer) using freshly collected material, but also from material that may be degraded whilst in transit to testing institutions. Although there were a few samples that did not readily amplify using this rapid detection protocol, with multiple samples and with including fresh and dried samples in assessments, the results are highly repeatable. Also, it was shown that the protocol developed here was highly sensitive to direct PCR using fungal mycelia without the labour-intensive and often costly process of DNA purification and sequencing, decreasing time and costs for positive identification of *F. euwallaceae* for monitoring purposes.

A less destructive method of sample collection than removal of bark to reveal lesions in sapwood is often required for high-value trees. We therefore tested the use of a small drill bit to collect sawdust from infected woody material for testing the rapid detection method developed here. Even though amplification of *F. euwallaceae* targeted DNA was less reliable using only

material collected using a drill bit, at least one out of five samples from each host species (barring English oak) produced detectible products. This method may however still be used when collecting samples from a fairly large number of entrance holes. Alternatively, a two-pronged approach using saw dust of diseased tissues and obtaining fungal cultures from these may be used in conjunction with the rapid detection technique proposed here and warrants future investigation. This can also be particularly useful if it is difficult to discriminate diseased tissues from healthy tissues or if oxidation of healthy tissues occurs rapidly after sampling, making discrimination between healthy and diseased tissues difficult.

In the present study, English oak was the only host to not have any samples that produced lysate with detectable *F. euwallaceae* DNA, despite the presence of *F. euwallaceae* being confirmed from all collected samples of this species. It is likely that some tree species, and possibly English oak, will have a very high concentrations of phenolic and other compounds that will inhibit the DNA polymerase enzyme during PCR (Peterson *et al*., 1997). It was noted that the lesions caused by *F. euwallaceae* in this host were much wetter and darker stained (nearly black) compared to the other hosts (drier lesions with light to dark brown or pink staining) and could therefore have contained high concentrations of secondary metabolites that may inhibit PCR. However, for hosts in which colonization by *F. euwallaceae* is suspected, but detection of fungal DNA from wood is not possible when using the rapid detection technique developed here, mycelia of isolates obtained from diseased tissues of hosts may still be used for rapid assay.

Some hosts that were dried prior to lysate preparation produced more readily detectable PCR product than their fresh counterparts, specifically bugweed, American sweetgum and pin oak. Bugweed contain strong odours (some of which may have PCR inhibitory properties) when fresh which reduces strongly after drying. However, fresh lysate from other hosts such as London plane and coastal coral trees led to more sensitive reactions than their dried counterparts. Sample preparation can therefore influence the sensitivity of the reaction and one would either need to test this before processing multiple samples from a specific tree species or samples need to be processed for both fresh and dried testing.

Because the PCR marker used in this study is species specific, there is a low risk of misidentification due to culture contamination by secondary microorganisms (Short *et al*., 2017). This makes this method particularly useful when working with environmental samples that typically contain many microbial taxa. Monitoring of *F. euwallaceae* on hosts using the technique developed here is therefore practical, fast and cost effective. But this technique may also be used to detect *F. euwallaceae* from other environmental samples such as beetle mycangia (e.g., crushed beetle heads; Kasson *et al*., 2013). It should be cautioned that where monitoring results of suspected PSHB infestation does not positively identify *F. euwallaceae*, traditional fungal isolation and subsequent identification should be carried out. While PSHB vectors only *F. euwallaceae* in South Africa, introductions of other ambrosia beetles vectoring plant pathogenic strains of fungi may facilitate PSHB acquisition of new fungal symbionts as

fungal promiscuity has previously been observed in the native region of PSHB (Carillo *et al.,* 2019). These same techniques may be used and modified to rapidly characterise the biology, ecology and vector relationships between closely related beetles from the *E. Fornicates* species complex and the various *Fusarium* taxa that they associate with in native and invaded regions. PCR markers specific to *Fusarium* spp. part of the AFC, AF-3, AF-4, AF-6, AF-8 and AF-8, which demonstrate high sensitivity have already been developed by Smith *et al*., (2019). For this, genomic data of all taxa (beetles and fungi) can be used to identify and develop species-specific markers that can be used in multiplex reactions to detect the various taxa from a multitude of different environments (Smith *et al.,* 2017; Vázquez-Rosas-Landa *et al*., 2021).A pipeline similar to the approach used by Smith *et al.,* (2017) to obtain markers for various taxa that form part of the AFC has been described by Vazquez-Rosas-Landa *et al*., (2021 which can be used for this exact purpose.

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Table 1: Summary of PCR results from *Fusarium euwallaceae*-colonised avocado tissues (lesion) and negative controls (no lesions) when varying the volume of polyvinylpyrrolidone (PVP) that was added to reaction mixtures. Included also are results obtained when lowering the lesioned woody material to half of the total tissues for lysate preparation. ++ = bright band, + = faint band, - = no amplification.

Volume of 2% PVP added	+ Control	Lesion	50% Lesion	No lesion
0 µl	++	-	-	-
1 µl	++	+	-	-
2 µl	++	++	-	-
3 µl	++	++	+	-
4 µl	++	++	-	-
5 µl	++	++	-	-

Table 2: Summary of PCR results from *Fusarium euwallaceae*-colonized plant tissues (lesion) and negative controls (no lesions) obtained from either fresh samples or the same samples dried before use. Included also are results obtained when lowering the lesioned woody material to half of the total tissues for lysate preparation. ++ = bright band, + = faint band, - = no amplification, +C = positive control.

			Fresh material			Dried material		
Host	**Sample #**	**+C**	**Lesion**	**50% Lesion**	**No lesion**	**Lesion**	**50% Lesion**	**No lesion**
Avocado	1	++	++	+	-	++	+	-
	2	++	++	+	-	++	+	-
	3	++	++	++	-	++	+	-
	4	++	++	+	-	++	++	-
	5	++	++	+	-	++	++	-
English oak	1	++	-	-	-	-	-	-
	2	++	-	-	-	-	-	-
	3	++	-	-	-	-	-	-
	4	++	-	-	-	-	-	-
	5	++	-	-	-	-	-	-
Lombardy poplar	1	++	++	++	-	-	-	-
	2	++	-	-	-	++	-	-
	3	++	-	-	-	++	+	-
	4	++	++	+	-	++	++	-
	5	++	++	++	-	++	-	-
Black wattle	1	++	++	-	-	+	-	-
	2	++	++	++	-	++	++	-
	3	++	++	+	-	++	+	-
	4	++	++	-	-	++	+	-
	5	++	++	+	-	++	-	-
Boxelder	1	++	++	++	-	++	++	-
	2	++	++	+	-	++	++	-
	3	++	++	++	-	++	+	-
	4	++	++	++	-	++	-	-
	5	++	++	++	-	+	-	-
Bugweed	1	++	-	-	-	++	++	-
	2	++	++	-	-	++	++	-

			Fresh material			Dried material		
Host	**Sample #**	**+C**	**Lesion**	**50% Lesion**	**No lesion**	**Lesion**	**50% Lesion**	**No lesion**
	3	++	-	-	-	++	+	-
	4	++	++	+	-	++	-	-
	5	++	++	+	-	++	+	-
Pin oak	1	++	-	-	-	+	-	-
	2	++	-	-	-	++	+	-
	3	++	-	-	-	++	-	-
	4	++	++	-	-	-	-	-
	5	++	+	-	-	++	+	-
London plane	1	++	++	++	-	++	+	-
	2	++	-	-	-	-	-	-
	3	++	++	+	-	++	-	-
	4	++	++	-	-	-	-	-
	5	++	++	-	-	-	-	-
Coastal coral tree	1	++	-	-	-	-	-	-
	2	++	++	-	-	-	-	-
	3	++	++	-	-	-	-	-
	4	++	-	-	-	-	-	-
	5	++	-	-	-	-	-	-
American sweetgum	1	++	-	-	-	++	+	-
	2	++	++	+	-	++	-	-
	3	++	++	+	-	++	+	-
	4	++	++	-	-	++	+	-
	5	++	+	-	-	++	-	-
Cork oak	1	++	-	-	-	++	+	-
	2	++	+	-	-	-	-	-
	3	++	-	-	-	++	+	-
	4	++	++	-	-	++	-	-
	5	++	-	-	-	++	-	-

Table 3: Summary of PCR results from *Fusarium euwallaceae*-colonised plant tissues (lesion) and negative controls (no lesions) obtained from fresh samples collected using a drill bit. ++ = bright band, + = faint band, - = no amplification.

Host	Sample number					+ Control	No lesion
	1	2	3	4	5		
Avocado	++	++	++	++	++	++	-
Coastal coral tree	-	-	-	+	-	++	-
London plane	++	++	-	-	++	++	-
American sweetgum	-	-	+	+	-	++	-
Cork oak	-	-	-	+	-	++	-
English oak	-	-	-	-	-	++	-
Lombardy poplar	++	+	++	-	-	++	-
Black wattle	-	++	++	++	+	++	-
Boxelder	++	++	+	++	+	++	-
Bugweed	++	++	-	-	-	++	-
Pin oak	-	-	-	+	-	++	-

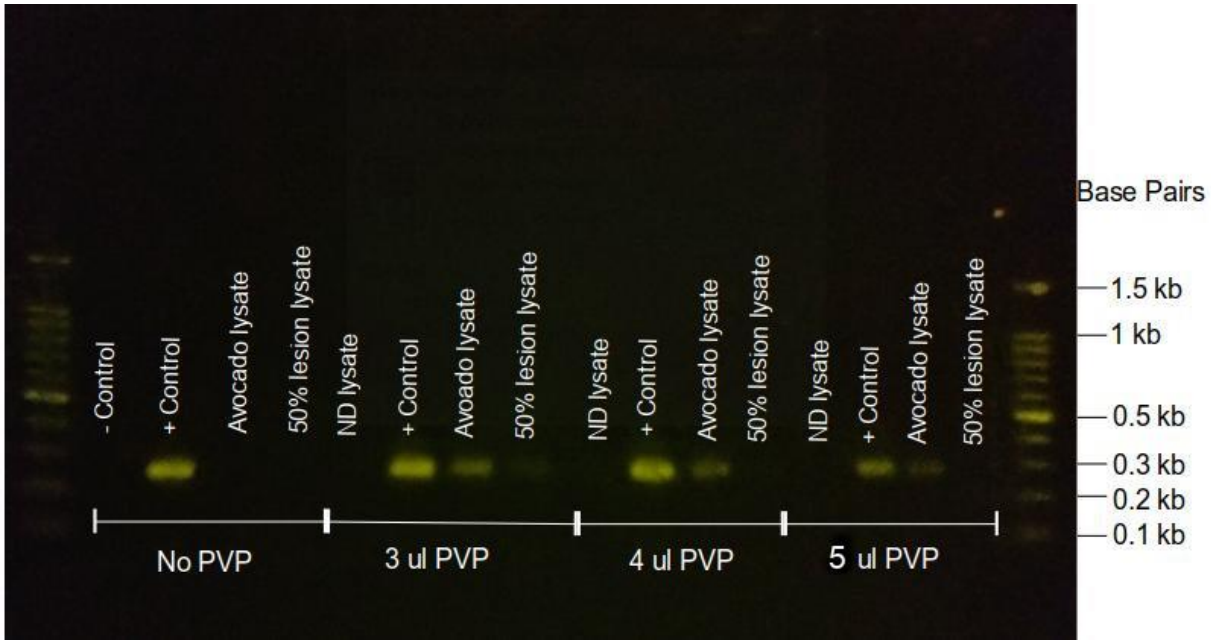

Figure 1: Image of gel electrophoresis demonstrating influence of different added volumes of polyvinylpyrrolidone (PVP) in PCR reaction mixtures on amplification of the target *Fusarium euwallaceae* gene fragment from lysate of diseased avocado woody tissue. Genomic DNA served as the positive control while no DNA/Lysate served as the negative control. Sensitivity of the PCR was tested by the addition of a sample that contained 50% diseased tissue and 50% healthy tissue.

CHAPTER 5: General conclusions

This study aimed to assess the pathogenicity of the ambrosia beetle *Euwallacea fornicatus* Eichhoff (Coleoptera: Curculionidae: Scolytinae) and its fungal symbiont *Fusarium euwallaceae* (Hypocreales: Nectriaceae) in agricultural production landscapes in wine grape vineyards and fruit orchards of plum, nectarine and apple. The study also set out to develop a rapid and low-cost identification protocol of *F. euwallaceae* in infected plants.

Two cultivars of grapevine were inoculated with three isolates and evaluated over the course of three months for disease progression. The lesions caused by *F. euwallaceae* were measured every 1-, 3- and 6-months post inoculation. There was no significant difference between the controls and plants inoculated with *F. euwallaceae* one month post inoculation, however some *F. euwallaceae* isolates were still recovered from the hosts. Three months post inoculation did not reveal any further disease development in either of the two cultivars. Furthermore no *F. euwallaceae* isolates were recovered from any of the grapevine stems harvested at this stage. Based on these results it was concluded that grapevine does not support *F. euwallaceae* growth, but viable spores/mycelia may remain within the entry holes a few weeks after initial attack. Grapevine has previously been reported as susceptible to Fusarium dieback, however the method with which symptom expression was characterized was incomplete as plants were not destructively analyzed and the presence of vascular lesions has not previously been reported. Isolations of *F. euwallaceae* made from previous observations were likely due to lingering spores/mycelia within tunnels excavated by the beetle. Since the beetle attacks hosts indiscriminately, it is possible that the PSHB reports on grapevine was an event due to chance alone. However, even though current findings suggest grapevine is resistant to *F. euwallaceae*, pathogen virulence or host susceptibility may be influenced by the environment. This study was carried out in a single production area within one region of the Western Cape. Previous studies found that the surrounding environment may influence the virulence of *F. euwallaceae* and grape vineyards should continue to be monitored for beetle colony establishment.

The effect of *F. euwallaceae* infection in plum, nectarine and apple trees was also evaluated by inoculating trees with fungal isolates confirmed to be *F. euwallaceae.* Results showed significant differences in length of lesions caused by *F. euwallaceae* and controls and the fungus was isolated from all trials, confirming pathogenicity of *F. euwallaceae* to all hosts included in this study. Nectarine trees showed increased susceptibility to *F. euwallaceae* when comparing lesion lengths to those found in plum trees. Data from monitoring reports and previous reports of beetle colony establishment has shown many species of *Prunus* to be susceptible to developing Fusarium dieback and are also reproductive hosts of PSHB. Based on the findings in this study and previous reports, stone fruit production may be particularly

susceptible to developing Fusarium dieback and presence of PSHB in orchards should be carefully monitored. Based on first reports here of PSHB reproduction in apple and data from many trees showing significant lesion development by *F. euwallaceae*, apple production may also be at risk of developing Fusarium dieback. Virulence of different isolates did not differ significantly indicating that the genetic variation of *F. euwallaceae* in South Africa might be quite small, as can be expected when initial colonization in the country was based on only a few individuals of PSHB. However, there should still be constant evaluation of fungal symbionts of PSHB, whether *F. euwallaceae* or other species, as shifts in fungal symbionts is still a possibility which may increase virulence.

Host response to increased attack was evaluated by comparing the average lesion length of branches inoculated with 1, 3, 5, or 7 holes. There was no difference in lesion data when comparing the different number of inoculation points on branches. The holes were drilled into the host at fixed lengths, while realistically the beetle would continue to excavate galleries throughout the host and continually inoculate the xylem with the fungus, especially if it is able to establish colonies. Enhanced diseased expression with an increase in colonization events may therefore still be observed in such cases. Therefore, to properly characterize disease expression on hosts, multiple inoculations may be useful. However, to evaluate susceptibility of hosts to *F. euwallaceae*, a single inoculation point is sufficient. The relationship between branch diameter and growth of *F. euwallaceae* was not different, indicating that the fungus can grow equally well in young and older plant tissues.

Comparisons of cultivar response was evaluated by comparing lesion data collected from two plum cultivars that were incubated for the same duration post inoculation. In this trial no significant difference was observed between the two cv. ‘Sungold’ and ‘Fortuner’, however this single trial is not necessarily representative of the phenomenon. Previous studies have found differences in susceptibility to developing Fusarium dieback between cultivars of avocado. It is recommend that further studies on other cultivars of stone fruit should be carried out as the differences in susceptibility would provide valuable information to future resistance breeding strategies. Because nectarine showed a higher susceptibility to *F. euwallaceae* when compared plum incubated for the same duration, susceptibility across stone fruit species should be evaluated do determine the overall risk across all taxa. Field observations showed that no external symptoms of disease development had occurred, despite very rapid growth of the fungus in some of the hosts evaluated in this study. This points to the urgency of pruning branches as soon as signs of beetle entry holes are observed. Future resistant variety development would be driven by studies of physiological traits that may be associated with disease resistance. Previous studies have also identified virulence genes of *F. euwallaceae*. By characterizing the infection process, identification of host genes of resistant varieties involved in

immunity can lead to improved resistance in fruit tree varieties and subsequent disease management in the future.

This study also aimed to establish a protocol for rapid detection of *F. euwallaceae* directly from symptomatic tissues. The purpose of this protocol was to expand monitoring capacity of PSHB and create a low-cost approach to pathogen diagnosis. A species-specific PCR marker for *F. euwallaceae* was identified for use in establishing this protocol. Optimization of PCR conditions showed that inclusion of 3 µl of a 2% (wt/v) polyvinylpyrrolidone (PVP) solution in the PCR mixture and adjusting the ddH_2O volume of the mixture resulted in optimum amplification of lysate prepared from grinding 0.015 g avocado lesion in 150 µl of TE buffer. To explore the reproducibility of this method, field samples were collected from ten other host species (English oak, Lombardy poplar, black wattle, boxelder, bugweed, pin oak, London plane, coastal coral tree, American sweetgum and cork oak) suspected to be infested with PSHB. These were evaluated using the optimised PCR conditions and lysate preparation from diseased tissues. To assess the effect of sampling on the sensitivity of the PCR reaction, fresh samples, samples that have been dried over the course of two days and samples that have been collected using drill sawdust from sapwood lesions were used to prepare lysate and subsequently used for PCR. Detectable PCR product was observed in all hosts included in this study besides English oak. Lysate prepared from fresh lesions compared to dried lesions did not perform better overall, however, dried lesions from some hosts such as American sweetgum and pin oak were more readily amplified than when lysate was prepared from fresh lesions from those hosts. However, London plane and coastal coral tree produced lysate that had more detectable fungal DNA when prepared from fresh lesions than from dry. There is no reliable way to determine which hosts will readily amplify using this protocol on wet or dried material, however including multiple samples will increase the outcome of positive PCR results. Lysate prepared from drill-bit collected sawdust also had detectable levels of fungal DNA in all samples tested except for English oak, although less samples amplified or produced thick bands during electrophoresis when compared to lysate prepared from shavings. Overall, though, this less destructive method of sampling is still highly applicable in cases where there is no other alternative. Isolates prepared from the hosts included in this study were used in the rapid detection protocol using the same procedure and had a 100% amplification rate. Mycelia used to prepare lysate from isolates is therefore a promising approach to correct false negatives where PSHB infestation is suspected. This is also especially helpful in hosts such as English oak, which could not be amplified regardless of nucleic acid preparation procedure. This is because *F. euwallaceae* isolates could still be recovered from diseased English oak tissues. Further exploration of this tool should include lysate preparation from crushed beetle heads to detect fungal spores located in the mycangia. The protocol developed here can be also applied to monitoring of other beetles and their fungal associates of the *E. fornicatus* species complex, thereby

facilitating characterisation of their biology, ecology and vector relationships. By designing species-specific primers for other plant-inhabiting diseases this approach could even be expanded to identification of other microbial taxa of interest from environmental samples.