**Screening for susceptibility of Macadamia to *E. fornicatus* and its fungal symbiont *Fusarium euwallaceae*.**

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## Abstract

The polyphagous shothole borer (*Euwallacea fornicatus*, PSHB), an ambrosia beetle, with its fungal symbiont, *Fusarium euwallaceae*, is responsible for Fusarium Dieback (FD) in a wide range of woody hosts. In 2019, the first suspected case of *E. fornicatus* was reported in macadamia in South Africa. The aims of this study were to confirm the *E. fornicatus* report and thereafter to assess the susceptibility of commercially planted macadamia cultivars to FD caused by *F. euwallaceae.* The identities of the beetle and associated fungal symbionts were confirmed by means of DNA sequence analysis of the 28S ribosomal large subunit gene for beetles and the internal transcribed spacer region for fungi. Isolates identified as *Fusarium* species were further characterised by phylogenetic analysis of the translation elongation factor 1α and the β-tubulin gene regions. Thereafter, Koch's postulates regarding *F. euwallaceae* were fulfilled on a mature *Macadamia integrifolia* tree planted at the experimental farm of the University of Pretoria. In order to determine susceptibility against FD, additional cultivar screening was conducted on nine commercially planted cultivars by means of pathogenicity trials using sterilized or inoculated toothpicks inserted into detached branches. Detached branch inoculations showed no significant lesion development six weeks post inoculation, except for cultivar 816. The restricted growth of *F. euwallaceae* observed in macadamia tissues therefore suggests that macadamia may not be a suitable host for *F. euwallaceae* and that the threat of FD in macadamia in the event of *E. fornicatus* infestation is less than for other *E. fornicatus* hosts. Future work on beetle attraction to macadamia is recommended for a more comprehensive understanding of the interaction between *E. fornicatus* and its fungal symbionts and macadamia.

## Main body

The polyphagous shothole borer (PSHB), *Euwallacea fornicatus* and its fungal symbionts have emerged as potential threats to a wide range of woody plants (Eskalen et al., 2013;

Mendel et al., 2017). From its place of origin in Southeast Asia, *E. fornicatus* has been reported in numerous areas around the globe including California (United States), Brazil, Israel and South Africa (Eskalen et al., 2012; Mendel et al., 2012; Paap et al., 2018; Smith et al., 2019). In South Africa, *E. fornicatus* has been confirmed on close to 100 species. In urban areas, the most seriously affected species include *Quercus robur* (English oak), *Acer negundo* (Boxelder), *Acer buergerianum* (Chinese maple) and *Plantanus* x *acerifolia* (London plane). In agriculturally important species, orchard infestation has only been reported for *Carya illinoinensis* (pecan nut) in South Africa although reports of *E. fornicatus* on *Persea americana* (avocado), *Prunus persica* (peach) and *Psidium guajava* (guava) trees have been confirmed in private gardens (van den Berg et al., 2019).

Host species in which the beetle is able to establish a gallery and survive but not successfully reproduce are known as non-reproductive hosts, while host species in which successful reproduction occurs are known as reproductive hosts (Eskalen et al., 2013, 2012). The reproductive success of the beetles is thought to be linked to the susceptibility of the host to colonization by the fungal symbionts which are also the primary food source of the beetles (Freeman et al., 2012). This was clearly demonstrated in a feeding study in which *Fusarium euwallaceae* was the only *Fusarium* species that allowed for the successful completion of the beetle's lifecycle (Freeman et al., 2012) alongside *Graphium euwallaceae* (Freeman et al., 2015). *F. euwallaceae* is also the causal agent of Fusarium Dieback (FD) in a number of tree species, with the fungus growing into neighbouring wood tissue, blocking xylem vessels and obstructing water flow throughout the plant. Fusarium Dieback can lead to major economic losses, due to removal and replacement cost of urban trees, or yield losses in agricultural sectors (Eskalen et al., 2013; Jones and Paine, 2017).

A better understanding of the interaction between macadamia, *E. fornicatus* and its fungal symbionts is required. Eskalen et al. (2013) isolated *F. euwallaceae* from wood tissue of infested *Macadamia integrifolia* and recorded macadamia as a non-reproductive but susceptible host. Mendel et al. (2017) also recorded the presence of *E. fornicatus* on *M. integrifolia* however was unable to isolate the fungus from xylem tissue. In 2019, the first suspected case of *E. fornicatus* was reported in macadamia in South Africa. The aims of this study were to confirm the identity of the beetle, to identify fungi isolated from beetles as well as beetle galleries, fulfil Koch's postulates regarding *F. euwallaceae*, and finally to assess the susceptibility of different commercial macadamia cultivars to FD caused by *F. euwallaceae.*

Macadamia branches that displayed beetle damage were collected from a commercial orchard in Marina beach, KwaZulu-Natal South Coast. Some branches were immediately sampled in order to isolate fungi while others were held in insect emergence chambers for 11 to 14 weeks. No distinct brown lesions developed on macadamia branches as is typical for *E. fornicatus* infestations on other hosts such as *Q. robur* and *Plantanus* x *acerifolia*. However multiple beetles that morphologically resembled *E. fornicates* did emerged from branches. A representative set of beetles were molecularly confirmed as *E. fornicatus.* This was done by means of DNA extraction using protocol outlined by Aljanabi and Martinez, (1997) with modifications that included centrifuging samples at 12 400 g instead of the proposed 1000 g and resuspending the final DNA pellet in 35 µL TE-buffer (10 mM Tris-Cl, 1 mM EDTA) for 2 h at 50°C. PCR amplification of the 28S ribosomal large subunit using primers 3665 & 4068 (Belshaw and Quicke, 1997; Cognato, 2013) was conducted at 95°C for 3 minutes followed by 35 cycles of 95°C for 30 seconds, annealing at 54°C for 30 seconds, elongation at 72°C for 1 minute, and a final elongation step at 72°C for 8 minutes. Sanger sequencing was conducted using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), the sequence Basic Local Alignment Tool (BLAST) searched against the NCBI database (www.ncbi.nlm.nih.gov) and uploaded to Genbank (MW004891).

Fungi were isolated from beetles and beetle galleries and pure cultures were obtained via single hyphal tip isolations. Isolates were grouped based on colony morphology and representatives of each were selected for molecular identification. DNA extractions were performed using the PrepMan™ Ultra kit (ThermoFischer Scientific). PCR amplification of the internal transcribed spacer region (ITS) was conducted using ITS1 and ITS4 (White et al., 1990) with thermal cycling at 95°C for 2 min, 35 cycles of 95°C of 30 sec, 55°C for 30 sec and 72°C for 30 sec, followed by 72°C for 1 min. PCR products were cleaned using ExoSAP-IT (Applied Biosystems, Foster City, CA) and sequencing of PCR products was conducted using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Identities of isolates were determined by Basic Local Alignment Tool (BLAST) searches against the NCBI database (www.ncbi.nlm.nih.gov). Isolates identified as *Fusarium* were further analysed by sequencing of the translation elongation factor 1α and the β-tubulin gene regions using TEF1 and TEF2 (O'Donnell et al., 2010) and T1 and T2 (O'Donnell and Cigelnik, 1997) respectively, with thermal cycling conditions as outlined by Herron et al. (2015). A Maximum Likelihood phylogenetic analysis of the EF1-α gene region was performed using MEGA7.5™. Additional sequence data included in the analysis was

obtained from Na et al. (2018), O'Donnell et al. (2015), Paap et al. (2018), van den Berg et al. (2019), as well as sequences of *F. euwallaceae* isolated from other hosts in South Africa.

The dominant species, obtained from beetles and beetle galleries were identified as *Fusarium* and the presence of *F. euwallaceae* from both beetles as well as the galleries were confirmed (Figure 1). The *F. euwallaceae* cultures were deposited in the CMW collection - Culture collection of Mike Wingfield (CMW55537, CMW55538, CMW55539, CMW55540 - FABI, University of Pretoria) and EF1-α gene region sequences used for phylogenetic analysis were uploaded to Genbank (MT043295, MT043296, MT043297, MT043298). Maximum Likelihood Phylogenetic analysis of the EF1-α gene region also confirmed the presence of a single *F. euwallaceae* haplotype, in agreement with all *F. euwallaceae* isolates obtained from South Africa thus far (Figure 1). Additional *Fusarium* species isolated from this study grouped within the larger Ambrosia Fusarium Clade (AFC), close to *Fusarium* 6 (AFC 6) (Kasson et al., 2013), one isolate was identified as *F. oxysporum* and the remaining isolates grouped with species within the *Fusarium incarnatum-equiseti* species complex (FIESC) (O'Donnell et al., 2009) (results not shown).

To fulfil Koch's postulates, three branches of a mature *M. integrifolia* at the experimental farm of the University of Pretoria, with diameters of 32 mm, 50 mm and 56 mm, respectively, were inoculated with *F. euwallaceae* CMW55537. Sterilized toothpicks were overgrown with the fungus for two weeks at 25°C and inserted into holes, 2.5 – 3 cm deep, drilled into the branches in a spiral-like fashion and secured with Parafilm™ as described by (Kusumoto et al., 2012). One sterilized toothpick per branch was inserted as experimental controls and a range of 3 to 8 inoculated toothpicks inserted per branch. For the branch of 56 mm diameter, holes were drilled at about 10 cm distance from each other whereas for the branches of diameters 32 mm and 50 mm, denser inoculations of 5 cm were used. This trial was conducted throughout spring and summer, Sept 2019 – Feb 2020, as this is the period when beetle activity and fungal growth is expected to be at maximum (Freeman et al., 2013; Umeda and Paine, 2019). At 12 and 23 weeks after inoculation of the live branches, no obvious lesion development was observed, however *F. euwallaceae* were re-isolated from tissue surrounding the inoculation sites at both time points.

Prior to conducting the commercial cultivar screening trial, a pilot study was conducted in order to determine the optimal assay to determine cultivar susceptibility. In the first study, one-year old macadamia cuttings were inoculated with two-week old *F. euwallaceae*

cultures by placing mycelial agar plugs (5mm in diameter) in wounds created within stems and sealed with Parafilm™. However, no lesion development was observed 6 weeks post inoculation. For the second assay, detached macadamia branches were inoculated with the toothpick method, similar as described for mature *M. integrifolia*, with a number of *F. euwallaceae* isolates, isolated from various hosts in South Africa (CMW52336, CMW51676, CMW52362, CMW53967 and CMW53966). Detached branches of a single cultivar, 695, were sealed at one end with wax and the other end allowed to stand in moistened sponges to imitate conditions in a living tree and were incubated for 6 weeks at 25°C ± 2°C. Eight replicates per isolate and 4 replicates with sterile toothpicks as experimental controls were included. Statistical analysis of lesion lengths was conducted using a Kruskal-Wallis test (Kruskal and Wallis, 1952) and a posthoc Dunn test using RStudio® Software (RStudio Team, 2019) and the R package  which showed significant lesion development for all *F. euwallaceae* isolates in comparison to the control inoculations ($\alpha = 0.05$; $H = 13.021$, $df = 5$, p-value = 0.023).

The commercial pathogenicity trial was subsequently conducted on detached branches, using the toothpick method, and included nine macadamia cultivars commercially planted by South Africa growers namely, 695, 344, 741, 788, 791, 800, 816, A4, and A16 (www.samac.org.za/industry-statistics). Branches with diameters ranging from 2 – 5 cm were inoculated with *F. euwallaceae* CMW55537 isolated from a macadamia tree and CMW53967 isolated from a pecan tree. A minimum of 5 replicates per isolate per cultivar were included as were 3 replicates per cultivar of sterile toothpicks as experimental controls. Six weeks after incubation under the same conditions as the pilot trial, lesion development was observed (Figure 2). A Kruskal-Wallis test was used to statistically analyse the results. No statistically significant lesion development was observed on any except the 816 cultivar ($H = 7.900$, $df = 2$, p-value = 0.019). Using the R package PMCMR (Pohlert, 2014), a posthoc Dunn test was then conducted showing that the pecan isolate, *F. euwallaceae* CMW53967, showed significant lesion development on cultivar 816 as compared to the control (Figure 2).

Lesion development on other known hosts of *E. fornicatus* have been reported at 5 cm in length two weeks post inoculation on *Plantanus* x *acerifolia* detached logs (Paap et al., 2018), 5 cm on *P. americana*, six weeks post inoculation (van den Berg et al., 2019), and lesions lengths of 10 – 12 cm, three weeks post inoculation also on *P. americana* (Eskalen et al., 2012). In comparison, a maximum lesion length of 1.7 cm was observed at 6 weeks

on the 816 detached branches whilst lesions were not observed on the living inoculated tree nor on infested branches despite beetle presence for up to 14 weeks. However, this may be linked to the status of macadamia as a non-reproductive host of *E. fornicatus* whilst both *Plantanus* x *acerifolia* and *P. americana* have been reported as reproductive hosts.

*Fusarium euwallaceae* does not seem to aggressively colonize macadamia wood tissue although its presence for up to 23 weeks without insect aid has been confirmed in this study. This apparent reduced susceptibility of macadamia, with little variation between cultivars, tested in the current study suggests FD due to PSHB is unlikely to progress quickly in healthy, and to a certain extent, in mildly stressed macadamia trees. However, future work should focus on the increase of beetle attacks recorded from macadamia orchards and the management of tree stress inducing factors that are the primary cause of secondary beetle attack. Future work on beetle attraction to macadamia is recommended for a more comprehensive understanding of the interaction between *E. fornicatus*, its fungal symbionts and macadamia.

## Acknowledgements

We acknowledge Macadamia South Africa NPC (SAMAC), The University of Pretoria (UP), the center of excellence in Plant Health Biotechnology (CPHB) and NRF Thuthuka for financial support. We also acknowledge Danroc that sponsored the seedlings used in the seedling pathogenicity trail as well as Mattison Farm that provided the detached logs used in the toothpick assay. Finally, we acknowledge Elisa Pal for her valuable contributions towards the statistical analyses.

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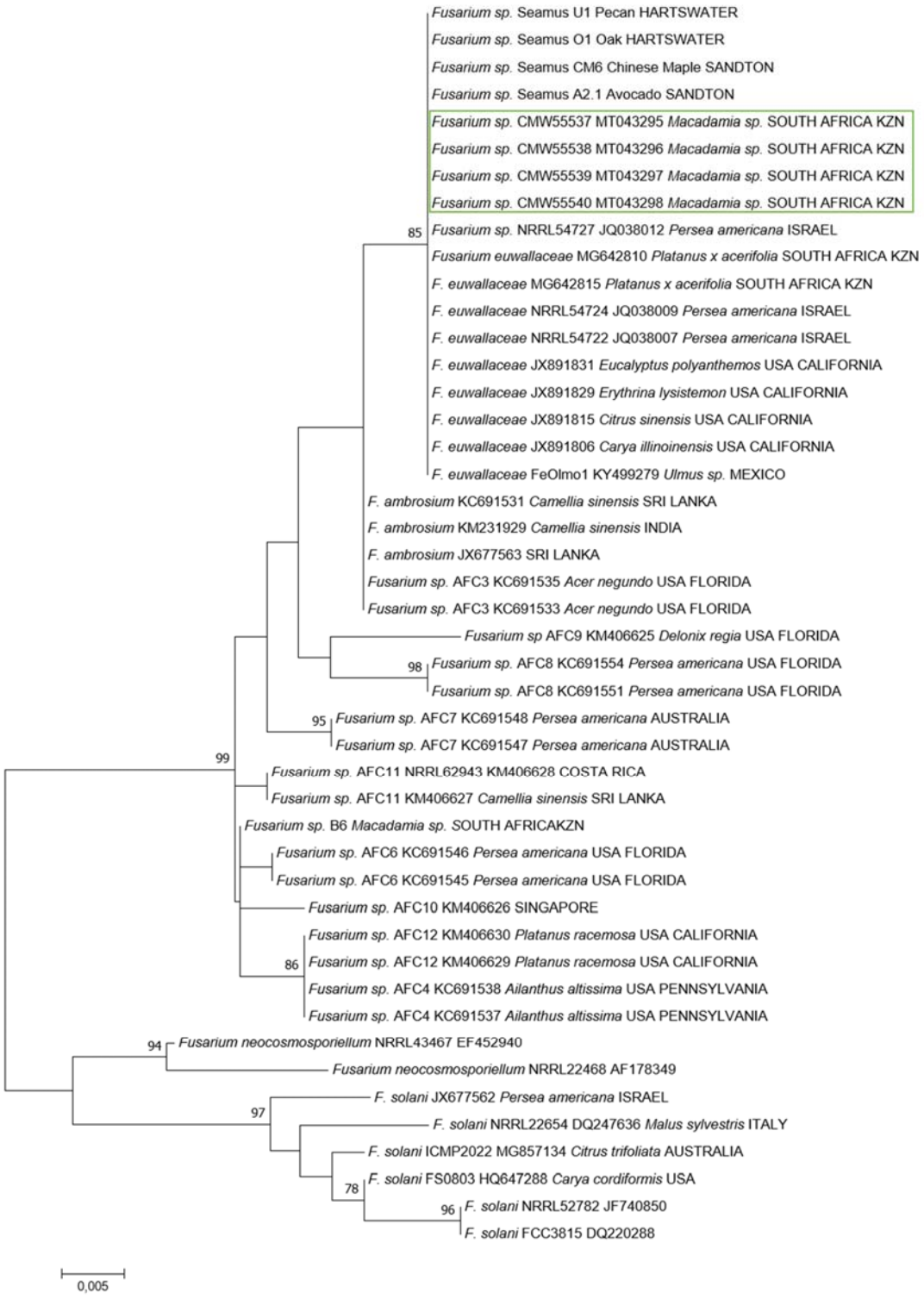


**Figure 1**. Maximum Likelihood phylogenetic tree of the translation elongation factor 1 – α gene region. Isolates obtained in this study from *Euwallacea fornicatus* beetles or infested macadamia host tissue, indicated in the green box, grouped closely with other *F. euwallaceae* isolates from both South Africa and other countries. This supports the identity of these isolates as *Fusarium euwallaceae*.

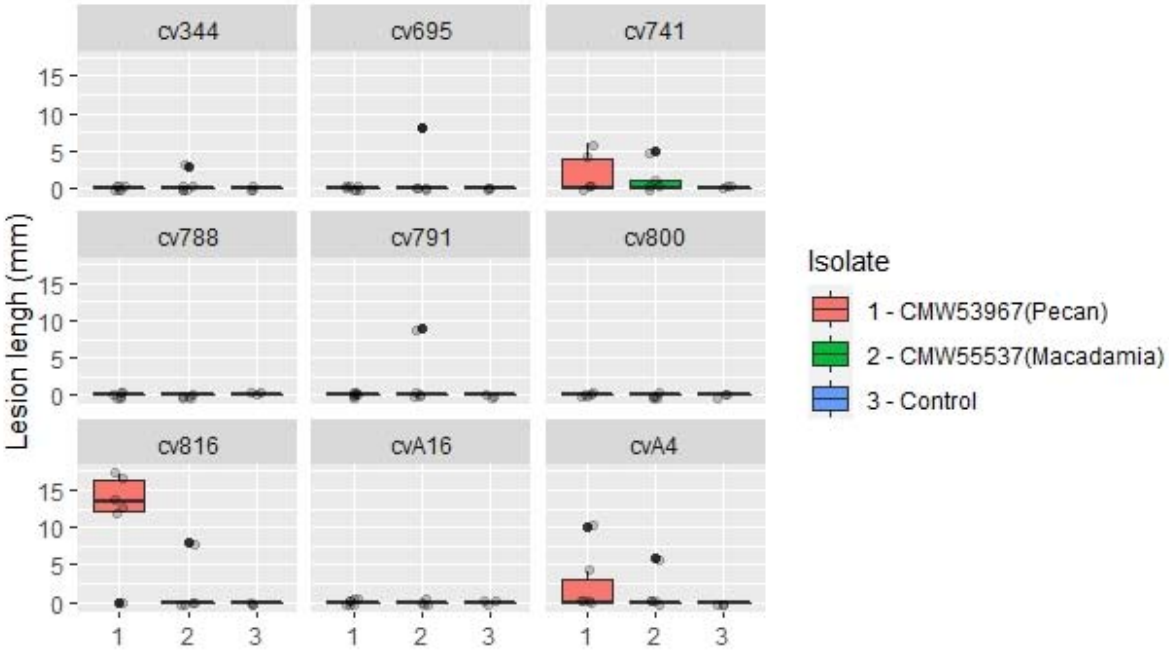


**Figure 2.** Lesion development observed from commercial macadamia cultivars, six weeks post inoculation. A Kruskal-Wallis test was used to statistically analyse the results. A posthoc Dunn test showed that the *F. euwallaceae* isolate CMW53967 developed significant lesion development on cultivar 816 as compared to the control.