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Biology and pathogenicity of fungi causing husk rot of macadamia in South Africa

Dee I. Twiddy · Arista Fouché · Olufemi A. Akinsanmi · Gerda Fourie

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Abstract Fungal diseases of macadamia fruit in South Africa have the potential to cause notable economic damage in this rapidly growing industry. To improve our understanding of the species involved in husk rot in macadamia orchards, a survey was conducted over two consecutive growing seasons to identify *Colletotrichum*, *Diaporthe* and *Calonectria* spp. that are associated with husk rot-infected macadamia fruits, and to investigate the occurrence of these fungi in asymptomatic and symptomatic fruits at the four stages of fruit development. Of the 425 fungal isolates obtained from the survey, *Colletotrichum* and *Diaporthe* were the most frequently isolated genera confirming the important role that these causal agents play in the husk rot epidemics. The detection of *Calonectria* species was low, only from symptomatic fruits and limited to a few locations in the main macadamia-producing provinces in South Africa. *Colletotrichum* and *Diaporthe* species were detected throughout the season at different stages of fruit development and in both symptomatic and asymptomatic fruits. The study confirmed that three fungal pathogens cause husk rot of macadamia in South Africa, with *Colletotrichum* and *Diaporthe* species that may have a latent phase in macadamia fruit. Studies of the growth characteristics of the husk rot pathogens revealed varied optimal growth temperatures, which may influence their prevalence in the different provinces in South Africa where macadamia is grown. The significance of the varied prevalence and biology of the causal agents in husk rot epidemics are discussed, which may be helpful management strategies.

Keywords Disease survey · Tree nut · Proteaceae · Epidemiology · *Colletotrichum* · *Diaporthe* · *Calonectria*

Introduction

Macadamia integrifolia Maiden & Betche, *Macadamia tetraphylla* L.A.S. Johnson, and their hybrids are cultivated worldwide. The production of macadamia nuts has steadily increased worldwide in recent years. In South Africa, a marked increase in new planting recorded is seen over the past decade (https://samac.org.za/information-hub). KwaZulu Natal, Limpopo and the Mpumalanga provinces are the dominant producing regions with minor plantings in the Western and Eastern Cape.

D. I. Twiddy · A. Fouché · G. Fourie (✉)
Department of Biochemistry, Genetics and Microbiology, Forestry and Agriculture Biotechnology Institute (FABI), The University of Pretoria, Pretoria 0002, South Africa
e-mail: gerda1.fourie@fabi.up.ac.za

O. A. Akinsanmi
Centre for Horticultural Science, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Ecosciences Precinct, GPO 267, Brisbane, Qld 4001, Australia

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The economic impact of diseases and insect pests is often related to nut quality after harvest. Available data from nut damage are mostly due to pests such as stink bugs (*Bathycoelia distincta* Distant (Pentatomidae)) (Schoeman, 2013; Sonnekus et al., 2022) and the macadamia nut borer (*Thaumatotibia batrachopa* Meyrick, 1908) (Smith et al., 2022), as well as kernel discoloration due to physiological factors (Le Lagadec, 2009) and immature kernels (https://www.worldmacadamia.com/resources). The impact of pre-harvest diseases on yield and kernel quality is challenging to quantify and is thus unclear. In addition, information regarding disease impact can only be achieved with sufficient knowledge of the disease and the pathogens involved. Research into fungal diseases of macadamia fruits has however increased in recent years, with several publications identifying causal agents in this previously understudied area (Akinsanmi & Drenth, 2017; Jiang et al., 2020b; Miles et al., 2009; Wrona et al., 2020).

Husk rot is the most widespread disease of macadamia fruits, classified by its symptoms that include soft brown to black necrotic lesions which can coalesce to cover the whole fruit in severe cases (Akinsanmi & Drenth, 2017; Jiang et al., 2020a; Wrona et al., 2020). Macadamia fruit consists of three layers, namely the pericarp or husk, shell, and the edible kernel. The pericarp or husk is the primary tissue through which nutrients are transferred to the enclosed kernel, ensuring the development and maturation of the nut (Strohschen, 1986). Infection of the husk tissue compromises nut development, often resulting in premature nut drop or preventing kernel maturation, thus negatively affecting yield and kernel quality.

Several fungal genera have been reported causing husk-rot like symptoms (Akinsanmi & Drenth, 2017; Chang et al., 2019; Fitzell, 1994; Jiang et al., 2019; Jiang et al., 2020a). The occurrence of husk rot symptoms associated with *Lasiodiplodia pseudotheobromae*, *Stibella* sp. and *Phytophthora heavea* in the orchards appears to be infrequent. *Colletotrichum gloeosporioides* sensu lato cause anthracnose husk rot (AHR) with orange concentric fruiting bodies that can form on the lesion surface under optimal conditions (Akinsanmi & Drenth, 2017; Fitzell, 1994; Hamilton & Fukunaga, 1959). Symptoms caused by *Diaporthe* species are termed Phomopsis husk rot (PHR) and include black shiny lesions without the formation of fruiting bodies (Akinsanmi & Drenth, 2017). More recently, *Calonectria pseudoreteaudii* syn. *pentaseptata* causing soft necrotic lesions on macadamia fruits were reported from China (Jiang et al., 2019, 2020b; Liu et al., 2020). One report described the disease as black spots which progressed into cracking of the husk with greyish-yellow mycelial growth (Jiang et al., 2020b), while the other described brown spots with white mycelial growth and a water-soaked boundary (Jiang et al., 2019). In 2017, *Calonectria* was also isolated from husk rot-like symptoms from macadamia orchards in the Limpopo province of South Africa. However, the disease ecology and identity of the species were not determined.

The behaviour of *Colletotrichum*, *Diaporthe* and *Calonectria* in other pathosystems may provide clues as to the disease cycle and likely methods of infection in the macadamia context. Lifecycles of species in the *C. gloeosporioides* species complex range from endophytic to pathogenic with a number of species exhibiting a hemibiotrophic lifestyle producing latent infections in a number of hosts (Jayawardena et al., 2021; Liu et al., 2022; Weir et al., 2012). The range of *Diaporthe* lifecycles is as diverse as *Colletotrichum*, which includes endophytes, saprobes, and highly aggressive pathogens with many species considered to be hemibiotrophs and some even considered as potential biocontrol agents against other pathogens (Ash et al., 2010; Debaeke & Moinard, 2010; Gomes et al., 2013). The *Calonectria* genus, on the other hand, consists of well-known aggressive pathogens that can persist within orchard soil and plant debris in the form of microsclerotia and chlamydospores without suitable conditions or hosts for infection (Aiello et al., 2022; Crous, 2002; Shishkoff & Camp, 2016).

The diverse lifecycle and ecology of *Colletotrichum*, *Diaporthe* and *Calonectria* highlight the need to properly investigate the species associated with husk rot of macadamia fruit in South Africa. The aim of this study was therefore to characterise the fungi associated with husk rot-infected macadamia fruits, and to investigate the prevalence of these fungi in asymptomatic and symptomatic fruits at four stages of fruit development. The role of temperature in the prevalence of the fungal pathogens causing husk rot was also investigated. Improving our understanding of the identity of the fungal species involved in the

incidence of husk rot in macadamia orchards in South Africa will contribute towards future management of the disease.

Materials and Methods

Survey and sample collection

Sampling of husk in the three main macadamia producing provinces in South Africa, namely Limpopo, Mpumalanga and KwaZulu Natal, was conducted from November to March of 2019/2020 and 2020/2021. As husk rot is often sporadic, structured sampling was not possible across the three provinces. Therefore, ten commercial macadamia orchards with a history of husk rot were surveyed, including two orchards in KwaZulu Natal, four in Mpumalanga and four in Limpopo. Köppen-Geiger climate zones in which these orchards were located include Cwb and Cwa in Limpopo, Cwa in Mpumalanga, and Cfb in KwaZulu Natal (Beck et al., 2018; Conradie & Kumirai, 2012).

In 2019/2020, ten symptomatic and asymptomatic fruits per tree were collected from five to ten trees of cultivars HAES 788, Nelmak 2, HAES 816, HAES 344, HAES 695 (Beaumont), HAES 814, and A4. These cultivars are the dominant macadamia cultivars in South Africa. In 2020/2021, sampling in two of the Mpumalanga orchards was also conducted on early season husk rot (November 2020), and late season husk rot, (February 2021), as well as a sampling of different stages of fruit development at a single time point (November 2020) (Supplementary Fig. 1). A total of 317 macadamia fruits were sampled from the three provinces during the two consecutive seasons. Samples were stored in brown paper bags at room temperature and processed within three days of collection.

Fungal isolation

Sampled fruits were surface disinfected in a 10% sodium hypochlorite solution for 2 min, 70% ethanol for 2 min and rinsed thoroughly with sterile water. Fungi were isolated from lesion margins and/or healthy husk tissue for asymptomatic fruits and plated on 2% malt extract agar (MEA) and pure cultures were obtained via single hyphal tip isolations. Isolates with colony morphology resembling *Colletotrichum* (Liu 2022; Weir 2012), *Diaporthe* (Gao et al., 2017; Gomes et al., 2013) and *Calonectria* (Crous et al., 2012, 2015; Pham et al., 2019; Rayner, 1970) were selected for molecular identification via DNA sequencing.

DNA extraction and PCR amplification

DNA was extracted from 7-day-old cultures using PrepMan™ Ultra (Thermo Fisher Scientific, Warrington, UK) according to the manufacturer's instructions. Multi-loci phylogenetic analyses of appropriate gene regions were used to identify *Colletotrichum, Diaporthe* and *Calonectria* isolates.

For *Colletotrichum,* amplified gene regions included the ITS gene region using primers ITS-1F (Gardes & Bruns, 1993) and ITS-4 (White et al., 1990), the partial beta-tubulin 2 (*tub2*) gene region using primers T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass & Donaldson, 1995), the glyceraldehyde-3-phosphate dehydrogenase (*gpdh*) gene region with primers GDF and GDR (Templeton et al., 1992), and the Apn2-Mat1 intergenic spacer and partial mating type Mat 1–2 region (Apmat) with primers AMF1 and AMR1 (Silva et al., 2012). For *Diaporthe* isolates, amplified gene regions included, the ITS gene region using primers ITS-5 and ITS-4 (White et al., 1990), *tub2* gene region with primers Bt2a and Bt2b (Glass & Donaldson, 1995), and the translation elongation 1- alpha (*tef*) gene region with primers EF1-526f and EF1-1567r (Rehner & Buckley, 2005).

For *Calonectria*, a partial beta-tubulin gene region (*tub2*) using primers T1 (O'Donnell & Cigelnik, 1997) and CYLTUB1R (Crous et al., 2004) was amplified to confirm genus and putative species complex via BLASTn analysis against the National Centre for Biotechnology Information (NCBI) database (Altschul et al., 1990). Representative isolates and additional gene regions were selected based on this information (Liu et al., 2020). Additional gene regions included the translation elongation factor 1 alpha (*tef*) using primers EF1-728F (Carbone & Kohn, 1999) and EF-2 (O'Donnell et al., 1998), the histone 3 gene region (*his3*) using CYLH3F and CYLH3R (Crous et al., 2004), the calmodulin gene region (*cmdA*) using CAL-228F (Carbone & Kohn, 1999) and CAL-2Rd (Groenewald et al., 2013) and

the actin gene region (*act*) using ACT-512F and ACT-783R (Carbone & Kohn, 1999).

Each PCR reaction included 0.2 µl of MyTaq™ DNA Polymerase (Bioline, South Africa), 5 µl of MyTaq™ DNA Polymerase Reaction Buffer containing $MgCl_2$ and dNTPs, 0.5 µl of each primer at 10 µM, 100 – 200 ng of template DNA and nuclease free distilled water (Adcock Ingram, Bryanston, South Africa) was added to a final volume of 25 µl. The PCR reaction conditions were 95 °C for 5 min, followed by 35 cycles of each primer pairs specific annealing temperature for 30 s (Supplementary Table 1), 72 °C for 1 min, with an elongation step of 72 °C for 10 min.

Amplification was confirmed and visualized via gel electrophoresis. PCR products were purified using ExoSAP-IT (Affymetrix Inc., California, USA). Sequencing reactions using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) were conducted and sequenced at the Bioinformatics Sequencing Facility at the University of Pretoria. All sequences generated in this study were submitted to GenBank (Supplementary Table 2).

Phylogenetic analysis

Consensus sequences were generated for all gene regions of each genus. Sequence alignments were assembled using MAFFT v. 7.505 (Katoh & Standley, 2013) and nucleotide substitution models determined with ModelTest-NG (Darriba et al., 2020). Reference and type sequences for phylogenetic analyses were obtained from the NCBI database based on published articles; for *Colletotrichum* from Liu et al. (2015), Liu et al., (2016) and Weir et al. (2012), for *Diaporthe* from Gao et al. (2016), Guo et al. (2020), Wrona et al. (2020), and Norphanphoun et al. (2022) and for *Calonectria* from Liu et al. (2020). Two isolates (CMW53649 and CMW53650) from the initial detection of *Calonectria* associated with husk rot symptoms in South Africa in 2017 were also included in the phylogenetic analyses.

Maximum likelihood (ML) analyses were performed using RAxML-NG (Kozlov et al., 2019) on CIPRES Science Gateway© (Miller et al., 2010) for both combined and individual gene regions with 1000 rapid bootstrap replicates. Before the concatenation of gene regions, a partition homogeneity test (ILD) was conducted on PAUP 4.0a169 (Swofford, 2003) to determine the congruence of the gene regions (Farris et al., 1995). Bayesian inference (BI) was conducted on the combined dataset using MrBayes 3.2.7a (Ronquist et al., 2012) on CIPRES Science Gateway©. Two runs of 4 Markov Chain Monte Carlo (MCMC) chains from random starting trees were conducted per analysis for 1 million generations with a partitioned GTR model. The heated chain temperature was set at 0.10 and the sampling frequency at every 500 trees. The first 25% of trees were discarded as burn-in and posterior probabilities determined with the remaining trees. Final consensus trees were viewed and annotated using iTOL (Letunic & Bork, 2021).

Pathogenicity trials

Pathogenicity trials were conducted on detached macadamia fruit obtained from orchards with little, to no history of husk rot. Fruits were examined for visible signs of fungal or pest damage and suitable fruits were surface sterilized as described for sampling.

Representative isolates for *Colletotrichum* (M001E4, M001C4, M001C2, M001B5), and *Diaporthe* (M001D4, M001D8, M001D9, M001C6) were used in pathogenicity trials on cultivars A4, Nelmak 2, and HAES 695 (Beaumont). Mycelial agar plugs 5 mm in diameter of *Colletotrichum* and *Diaporthe* were placed on five replicate wounded fruits per cultivar with a 5 mm diameter cork borer and sealed with Parafilm© to prevent drying out of the agar plugs. Clean agar plugs were used as negative controls.

For *Calonectria*, conidial suspensions of 10^6 cfu/mL of representative isolates (CMW60086, CMW60084, CMW60090, CMW60091, CMW60089, CMW60087) were prepared from 2-week-old cultures on MEA with 10% Tween20 solution. For one isolate (CMW60086), ten wounded and unwounded Nelmak 2 fruits were inoculated with a 10 µl conidia suspension to compare the effect on lesion development as described by Akinsanmi and Drenth (2017). Pathogenicity trials for the other isolates were conducted with wounding on ten replicate fruits. Mock inoculations with sterile water were conducted for all treatments as negative controls.

Fruits inoculated with *Colletotrichum, Diaporthe* and *Calonectria* were stabilized with sterilized cotton wool and incubated in a sealed container at high relative humidity and exposure to natural light at 25 °C ± 2 °C for 14 days. Thereafter, lesion

development was recorded, and re-isolation of fungi conducted to fulfil Koch's postulates where appropriate. A scoring system from 0 – 5 was used to categorize lesion severity by the percentage of husk surface with visible lesions. Each category represented a 20% increase in lesion coverage, i.e., 0 = 1 – 20% and 5 = 80 – 100%.

Temperature growth studies

Growth studies were conducted *in-vitro* with five replicates per isolate used in the pathogenicity trials at six different temperatures with 5 °C increments from 10 °C to 35 °C. Mycelial plugs of 5 mm in diameter were obtained from 7-day-old cultures and placed on MEA plates. The diameter of the colony growth was recorded every second day and the average growth rate per day was determined after 14 days. Cultures that showed no growth at the end of the trial were placed at optimal growth temperature, 25 °C, for a week to determine if the treatment temperature had a lethal effect on the fungus.

Statistical analyses

Ordinal data from the pathogenicity assays was analysed with the Kruskal–Wallis test (Kruskal & Wallis, 1952) and a Dunn's All-Pairs Rank Comparison Test (Dunn, 1964) using R v4.3.0 (RStudio Team, 2022) and R packages including PMCMRplus (Pohlert, 2022), Rmisc (Hope, 2022), and ggplot2 (Wickham, 2016). Average growth data was analysed using a one-way analysis of variance (Girden, 1992). Data visualization was done using RStudio 2023.03.1 + 446 (R Studio Team, 2022).

Results

Sampling and identification

A total of 317 macadamia fruits across ten orchards were sampled from which 425 fungal isolates were obtained representing 16 fungal genera (Supplementary Table 3). Isolate classifications were confirmed with BLASTn analysis (Altschul et al., 1990) of the ITS gene regions of *Colletotrichum* and *Diaporthe* and the *tub2* gene region for *Calonetria.* The most frequently isolated genera were *Colletotrichum* and *Diaporthe* followed by Botryosphaeriaceae, *Calonectria, Fusarium* and *Neopestalotiopsis* (Fig. 1). Across the three provinces surveyed, *Colletotrichum* was dominant in Mpumalanga (MP), *Diaporthe* more dominant in the KwaZulu Natal (KZN) and *Calonectria*

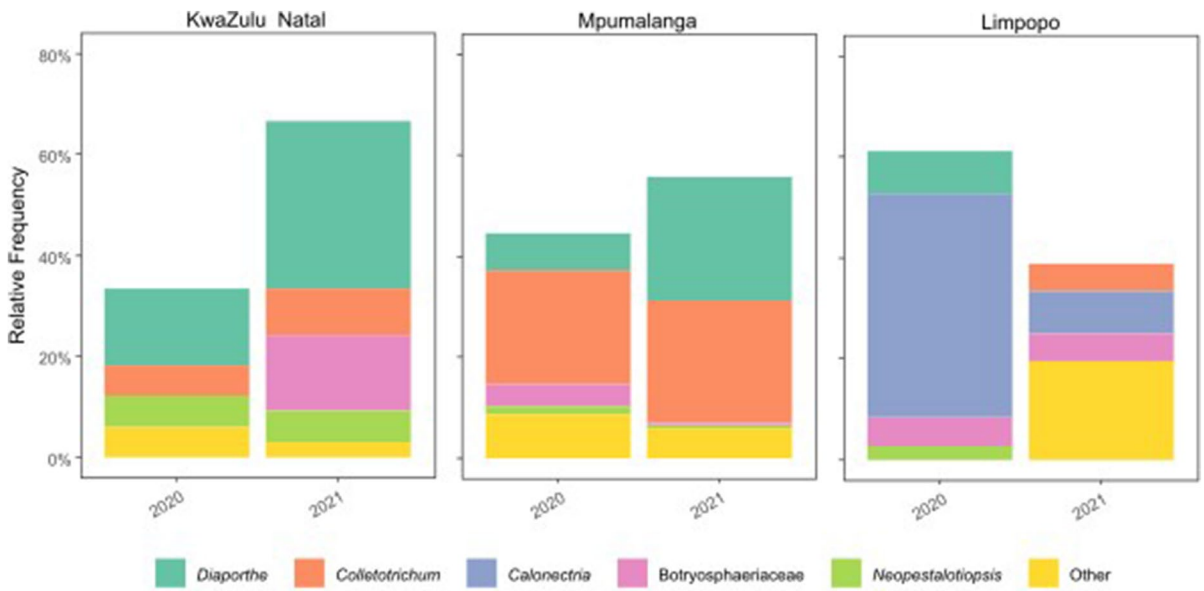

Fig. 1 Bar graphs showing the number of isolates per genus obtained from macadamia fruit by season and province representing the three main macadamia producing regions in South Africa

in Limpopo (LP) samples. However, a much smaller sample set was included for KZN and LP in comparison to MP, which may have skewed the prevalence of the genera.

Phylogenetic analyses

For selected *Colletotrichum* isolates, gene region length after alignment and trimming were 568 bp for ITS, 600 bp for *tub2*, 298 bp for *gpdh* and 929 bp for the ApMat region. A partition homogeneity (ILD) test of these gene regions revealed a level of congruence previously reported as acceptable for accurate phylogenetic analyses ($p=0.01$) (Cunningham, 1997; Farris et al., 1995). ML and BI analyses of the ITS, *tub2*, *gphd* and Apmat datasets revealed four species of *Colletotrichum* (Figs. 2 and 3). More than 58% of the selected isolates were identified as *C. theobromicola*, 27% were identified as *C. siamense*. The remaining isolates were split between *C. fructicola* and *C. alienum*.

For selected *Diaporthe* isolates, 542 bp for ITS, 491 bp for *tub2* and 379 bp for *tef* gene region were generated. An acceptable level of congruence between the three gene regions was revealed by an ILD test ($p=0.01$) (Cunningham, 1997; Farris et. al. 1995). ML and BI analyses of the concatenated gene regions placed all representative *Diaporthe* isolates within the *D. oncostoma* species complex. ML and BI analysis revealed most isolates (84%) grouped in a clade comprising of the species *D. nebulae*, *D. macadamiae*, *D. anacardii*, *D. velutina*, *D. portugallica* and *D. phillipsii*. Further species delimitation within this group had adequate BI support values (>0.80) but low support from the ML analysis (<75). Isolate M001D5 had very strong BI support for grouping near *D. phillipsii* and was allocated as such. For the rest of the group's isolates, BI analysis supported a closer relation to *D. nebulae* than the other species, and for the purpose of this study are referred to as such. The remaining six isolates grouped close to *D. baccae*, and a potentially novel species near *D. inconspicua*. As only two isolates were identified for each of these three species, this study focused mainly on the *D. nebulae* isolates hereafter.

For selected *Calonectria* isolates, 680 bp for *cmdA*, 440 bp for *his3,* 300 bp for *tef*, and 250 bp for *act* were generated. BLAST analysis of the *tub2* gene region (approx. 560 bp) confirmed all *Calonectria* isolates are in the *Ca. candelabrum* species complex, a different species complex to the *Calonectria* isolates identified in China. An acceptable level of congruence between these gene regions was revealed by an ILD test ($p=0.01$) (Cunningham, 1997; Liu et al., 2020; Pham et al., 2019). ML and BI analyses revealed three distinct clusters (Fig. 4). Three isolates from this study and the two isolates collected from Limpopo in 2017, formed a separate novel cluster (*Calonectria* sp. nov.1.) closest to *Ca. pseudospathulata* with adequate support from both ML and BI analyses. Another three isolates also formed a separate cluster closest to *Ca. pauciramosa*, with strong support for distinction from *Ca. pauciramosa* in the consensus tree and we therefore refer to these isolates as *Calonectria* sp. nov. 2. Finally, an additional two isolates from Mpumalanga were identified as *Ca. pauciramosa* as there is weak support for differentiation in the consensus tree.

Distribution in asymptomatic vs symptomatic fruits

Differences in the prevalence of fungal isolates on asymptomatic versus symptomatic macadamia fruit was observed in the first growing season (Fig. 5A). *Colletotrichum* was more frequently isolated from symptomatic ($n=61$) than asymptomatic ($n=22$) fruit. At a species level, *C. alienum* and *C. fructicola* were isolated from symptomatic fruits only. *Diaporthe*, on the other hand, was more frequently isolated from asymptomatic ($n=23$) than symptomatic fruits ($n=11$). *Calonectria* was the only group isolated exclusively from symptomatic fruits. Other genera such as the Botryosphaeriaceae were more often isolated from asymptomatic fruits except for the *Neopestalotiopsis* group with showed a greater portion of isolates from symptomatic fruits (Fig. 5A).

Distribution within season changes

An early and late sampling of husk rot symptoms in the second season revealed differences in fungi prevalence on symptomatic nuts within a single growing season (Fig. 5B). Early summer fruits (November) had an equal proportion of *Diaporthe* and *Colletotrichum* isolates, whereas later summer fruits (February) sampled in the same orchard rows had an increased proportion of *Colletotrichum*, representing 75% of isolates obtained.

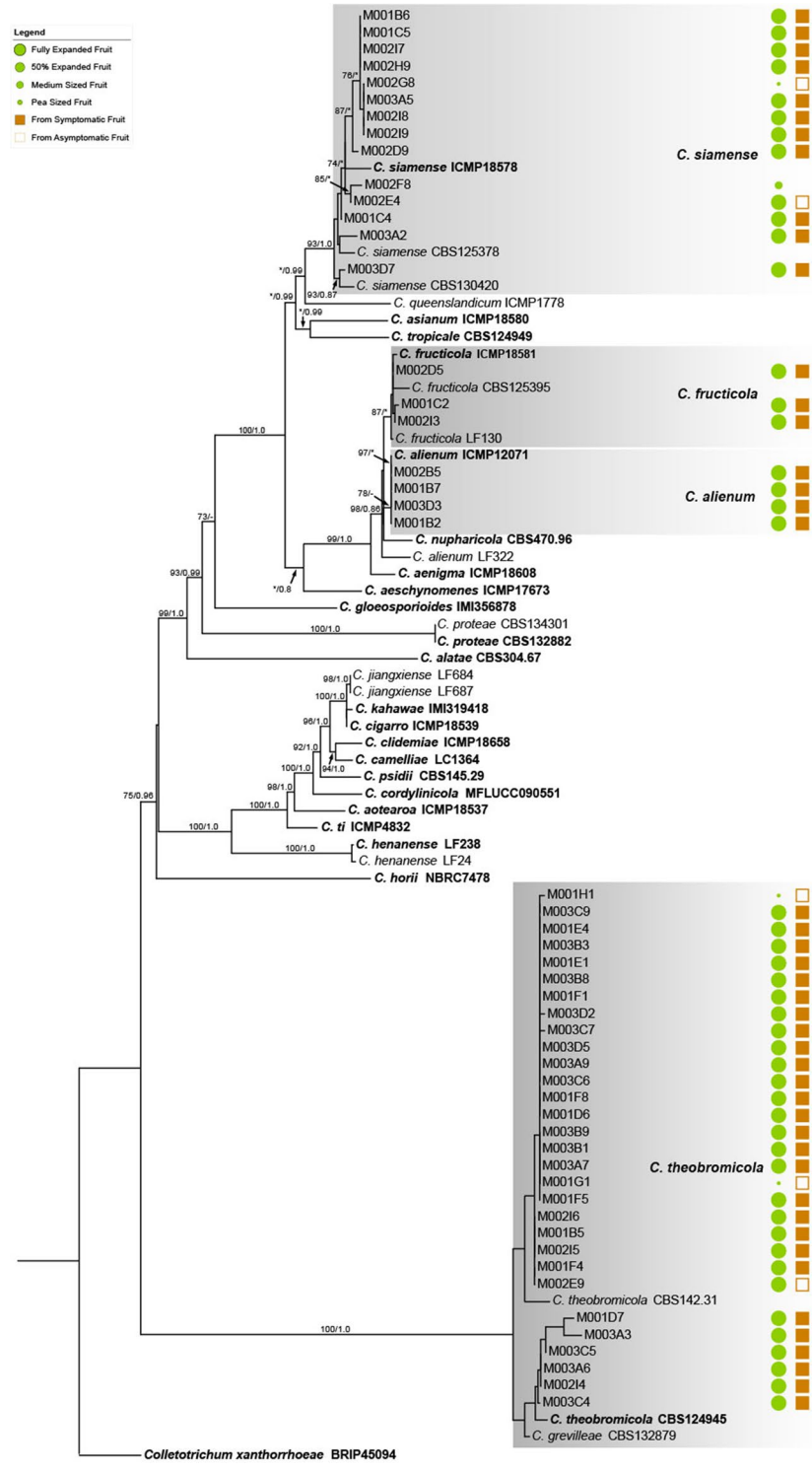

Fig. 2 Multi-locus phylogenetic tree maximum likelihood (ML) and Bayesian inference (BI) analyses of the ApMat, *its, tef, tub* gene regions of the *Colletotrichum* isolates including representative isolates from this study in bold. ML bootstrap values ≥ 70 and posterior probabilities of a Bayesian inference analysis ≥ 0.80 are shown at nodes; values lower than the cut-off are marked with "*"

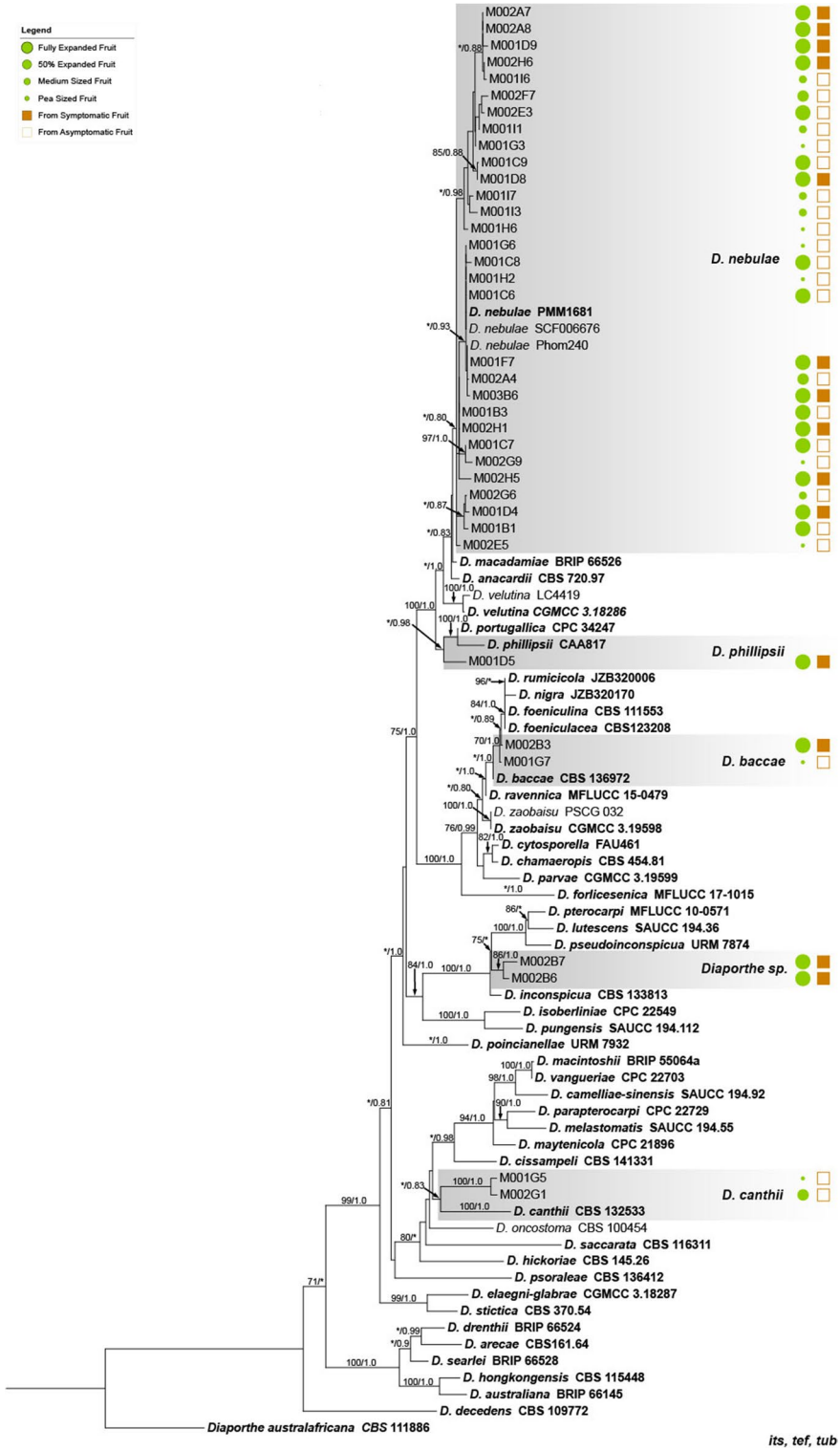

Fig. 3 Multi-locus phylogenetic tree maximum likelihood (ML) and Bayesian inference (BI) analyses of the *ITS, tef,* and *tub* gene regions of the *Diaporthe* species including representative isolates from this study in bold. ML bootstrap values ≥ 70 and posterior probabilities of a Bayesian inference analysis ≥ 0.80 are shown at nodes; values lower than the cut-off are marked with "*"

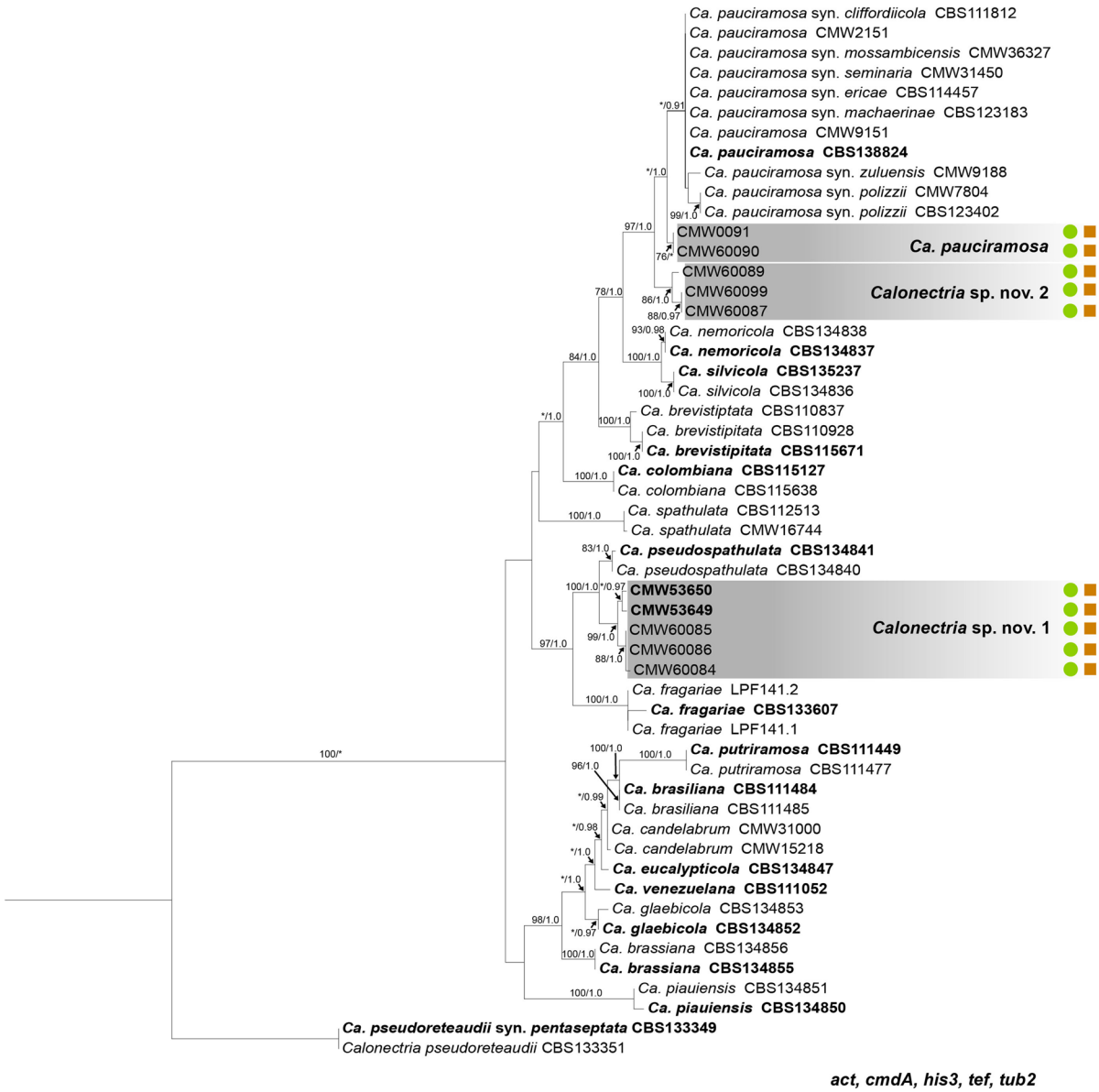

Fig. 4 Multi-locus phylogenetic tree maximum likelihood (ML) and Bayesian inference (BI) analyses of the *act, cmdA, his3, tef,* and *tub2* gene regions of the *Calonectria candelabrum* species complex including representative isolates from this study in bold. ML bootstrap values ≥ 70 and posterior probabilities of a Bayesian inference analysis ≥ 0.80 are shown at nodes; values lower than the cut-off are marked with "*". Isolates obtained in RSA in 2017 are indicated in bold

No *Calonectria* were detected since it was absent from the orchards selected for within-season sampling.

Distribution based on fruit developmental stages

Different phenological stages in macadamia fruit development, pea-sized, medium, 50% expanded and fully expanded, corresponded with differences in fungal prevalence (Supplementary Fig. 1). At the time of sampling, husk rot symptoms were only present on the fully expanded fruits in the orchard. Overall, *Diaporthe* was the most prevalent fungus on fruits throughout the developmental stages (Fig. 5C). Very low incidences of *Colletotrichum* were recorded from

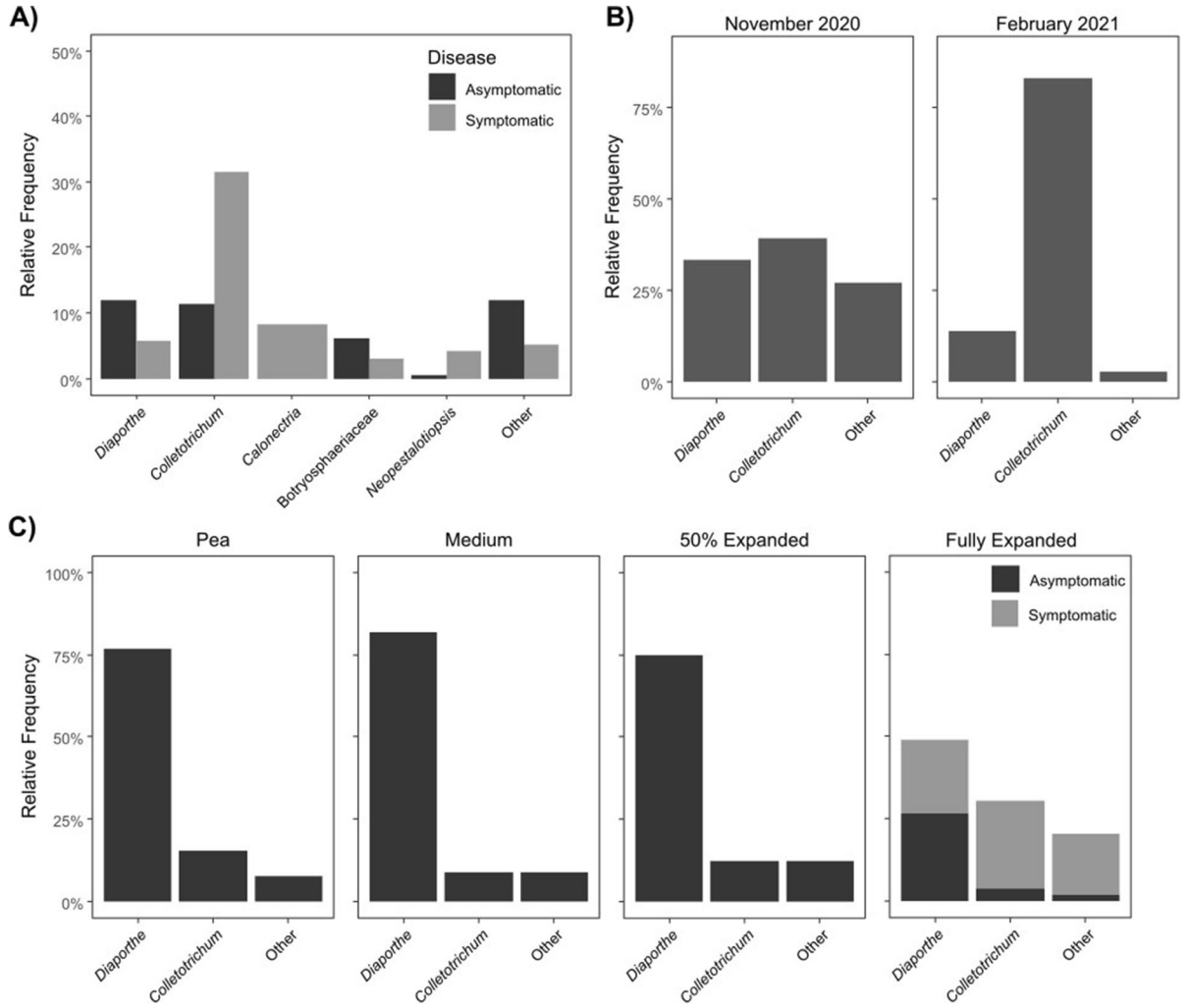

Fig. 5 Bar graph showing the percentage of isolates: (A) obtained from asymptomatic and symptomatic macadamia fruits during the first growing season (2019/2020), (B) obtained from husk rot symptomatic fruits at different time points (November and February) in the 2020/2021 season (C) obtained per genera from various fruit development stages sampled in November 2020. Darker colours indicate isolates obtained from asymptomatic fruits, lighter from symptomatic fruits

the smaller three sizes with a marked increase in the fully expanded fruits. Fully expanded fruits with husk rot symptoms had higher prevalence of *Colletotrichum* than *Diaporthe*. *Diaporthe* was however the dominant species detected in asymptomatic fully expanded fruits.

Pathogenicity assays

All *Colletotrichum, Diaporthe* and *Calonectria* isolates included in the assay were able to produce lesions on detached macadamia fruit. Fruits inoculated with *Diaporthe* isolates developed black to dark brown lesions typical of symptoms previously associated with PHR (Supplementary Fig. 2), with some raised fruiting bodies developing on severe lesions. Fruits inoculated with *Colletotrichum* isolates developed slightly more brown-coloured lesions with many developing orange fruiting bodies during incubation (Supplementary Fig. 3), typical of symptoms associated with AHR. Fruits inoculated with *Calonectria* developed symptoms that corresponded to field

observations in 2017, as dark brown to black lesions with white mycelial growth on the husk surface in the centre of the lesion (Supplementary Fig. 3). Some control fruits developed lesions away from the inoculation point likely due to latent infections. This mostly occurred on the fruits of the Beaumont (695) cultivar and all instances were included in statistical analyses of the results.

Diaporthe nebulae isolates produced significantly different lesion severities ($p < 0.05$, df = 3; Fig. 6A). Isolates M001D9 and M001D8 produced significantly severe lesions on Nelmak 2 fruits. Isolates M001D9, M001D8 and M001D4 produced significantly severe lesions HAES 695 (Beaumont) and A4 fruits. Isolate M001C6 produced significantly severe lesions on A4 fruits. Cultivar A4 reported the least severe lesions overall. *Colletotrichum* species also produced significantly different lesion severities ($p < 0.01$, df = 3; Fig. 6B). *C. siamense* and *C. fructicola* isolates produced significantly more severe lesions on cultivars HAES 695 (Beaumont) and Nelmak 2 than those produced by *C. theobromicola*. Cultivar A4 reported the lowest severity score with not significant difference between isolates.

In terms of *Calonectria*, significant lesion development was observed for *Calonectria* sp. nov. 1 (CMW60086) on both unwounded ($H(1) = 6.201$, $p = 0.012$) and wounded fruits ($H(1) = 16.844$, $p < 0.001$). No significant difference between symptom severity on wounded and unwounded fruits ($H(1) = 2.466$, $p = 0.116$) was observed, however the mean lesion severity was higher for the wounded fruits. Significant lesion development ($p < 0.05$) was also observed for all three species in subsequent pathogenicity assays (Fig. 7). *Calonectria* sp. nov. 1 caused significantly more severe lesions than *Calonectria*

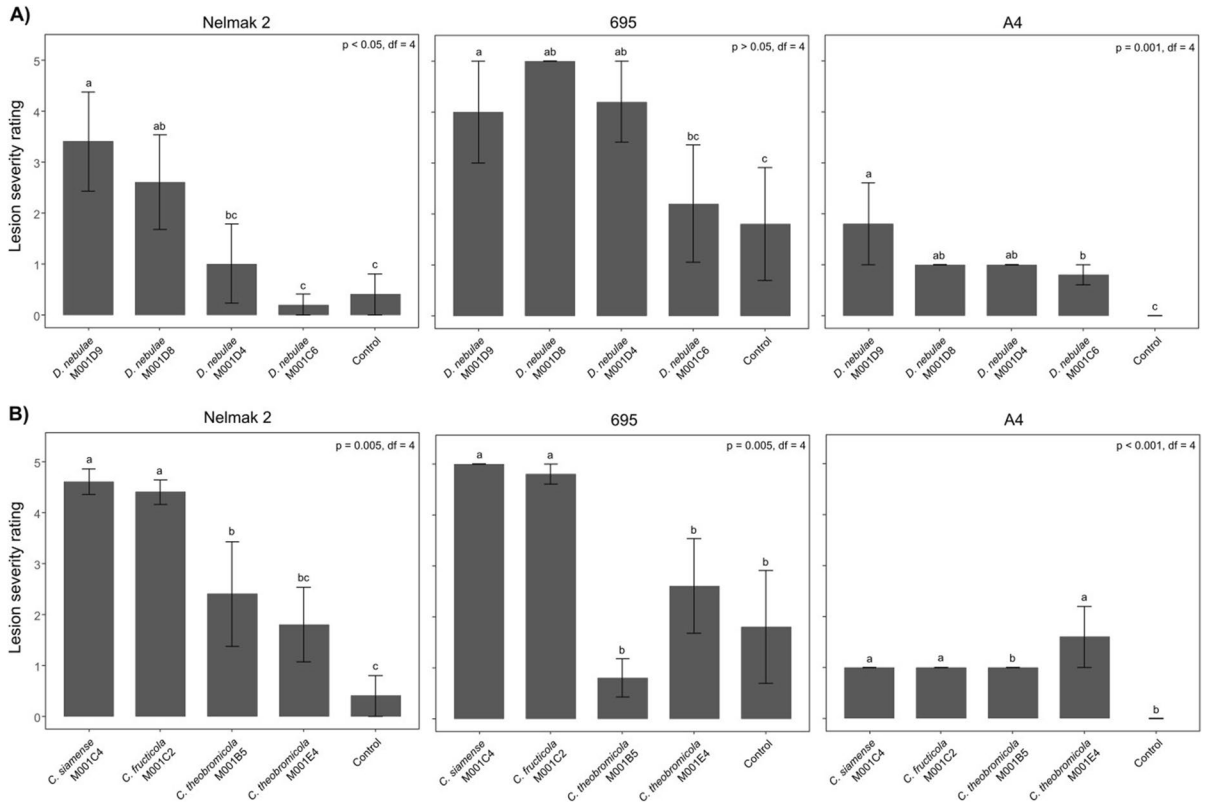

Fig. 6 Results of pathogenicity trials with *Diaporthe* (A) and *Colletotrichum* (B) isolates associated with husk rot disease of macadamia, 14 days after inoculation. Lesion severity was scored based on the percentage coverage of the husk by the lesion from 0 – 5 with 0 representing 0% and then increasing in increments of 20%. Significant differences between isolates were confirmed with a Kruskal–Wallis rank sum test and post-hoc Dunn test ($\alpha = 0.05$). Isolates with the same letters did not produce significantly different lesion severities

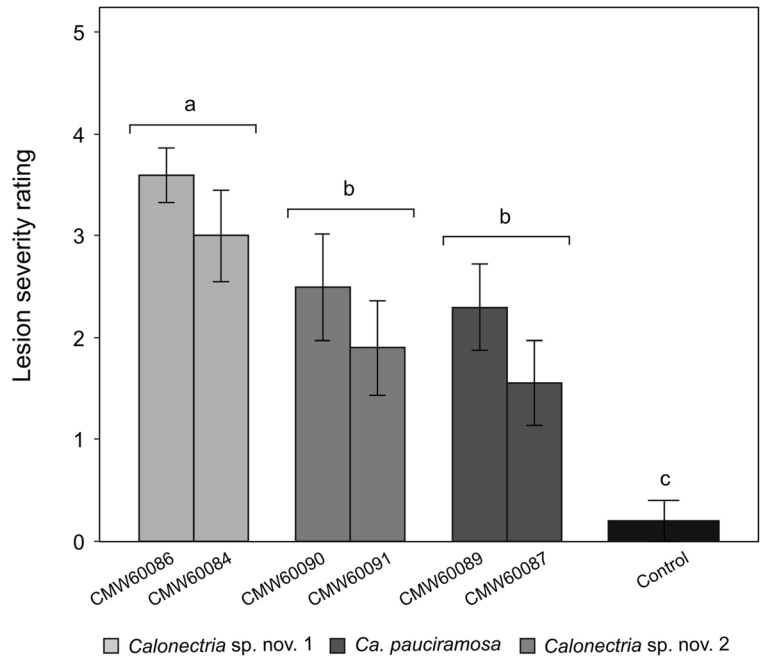

Fig. 7 Results of the pathogenicity trials 14 days after inoculation with *Calonectria* spp. and incubation at 25 °C ± 2 °C. Lesion severity was scored on a scale from 0 – 5. A Kruskal–Wallis test and post hoc test Boniferroni was conducted on the ordinal data set ($\alpha = 0.05$). Whiskers represent the standard error of the mean

sp. nov. 2 and *Ca. pauciramosa*. *Calonectria* isolates were confirmed as causal agents as they were re-isolated from lesions, fulfilling Koch's postulates.

Growth studies

The average daily growth rate at the six temperatures did not vary significantly between species for *Diaporthe* or *Colletotrichum* ($p > 0.05$), however overall growth rate was slower for all *Calonectria* isolates (Fig. 8). The optimal growth temperature for most isolates was 25 °C; *C. alienum* (5.0 mm day^{-1}), *D. nebulae* isolates (4.3 mm day^{-1}), *Calonectria* sp. nov. 1 (2.6 mm day^{-1}), *Calonectria* sp. nov. 2 (2.8 mm day^{-1}) and *Ca. pauciramosa* (3.0 mm day^{-1}). The optimal growth for three *Colletotrichum* species

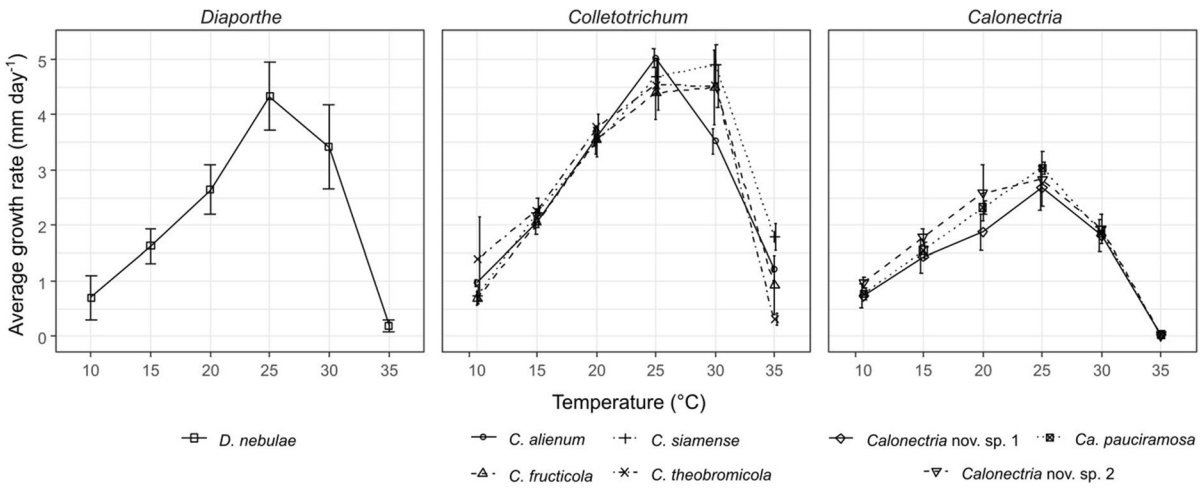

Fig. 8 Average daily growth rates for *Colletotrichum, Diaporthe* and *Calonectria* species associated with macadamia fruit at six temperatures ranging from 10 °C—35 °C

was recorded at both 25 °C and 30 °C, *C. fructicola* averaged at 4.4 mm day^{-1} ± 0.01 mm, *C. siamense* at 4.7 mm day^{-1} ± 0.2 mm, and *C. theobromicola* at 4.5 mm day^{-1}. The slowest growth rates were observed at 35 °C for *Diaporthe* and *Colletotrichum*, while 35 °C proved a lethal temperature for all *Calonectria* isolates after two weeks of incubation. The relationship between temperature and growth for the *Diaporthe*, *Colletotrichum* and *Calonectria* isolates tested is represented by a bell-curved with a left skew (Fig. 8).

Discussion

This study identified *Diaporthe* and *Colletotrichum* as the most dominant genera associated with macadamia fruits in South Africa, confirming the important role that these pathogens play in husk rot epidemics. At least four species in each of the fungal genus are associated with husk rot in macadamia. Trends regarding the prevalence of *Colletotrichum* and *Diaporthe* revealed a tendency for *Diaporthe* to be associated with macadamia fruit from early in a growing season, while the frequency of *Colletotrichum* increased as the fruit matures. In addition to *Diaporthe* and *Colletotrichum* spp., this study also confirmed *Calonectria pauciramosa* and two novel *Calonectria* species as husk rot pathogen of macadamia fruit in South Africa. The symptoms produced by the three fungal genera have distinguishing features that enabled distinct classification of the husk rot caused by *Calonectria* spp. as Calonectria husk rot (CHR). The frequency of CHR occurrence, in comparison to Colletotrichum husk rot (AHR) and Phomopsis husk rot (PHR), is however low and only occurred in symptomatic fruit. Although the formation of orange concentric fruiting bodies may not always present in-field to differentiate between AHR and PHR, CHR symptoms especially the white mycelial growth on the husk surface in the centre of the lesion are distinct.

The majority of the *Diaporthe* isolates identified in this study were all part of the *D. oncostoma* species complex (Norphanphoun et al., 2022) with BI analysis suggesting a closer relation to *D. nebulae* than *D. macadamiae*, a species that was previously described from macadamia orchards in South Africa (Wrona et al., 2020). *D. nebulae* was described from grapevines in the Western Cape province of South Africa in 2019 and is reported among the virulent species causing Phomopsis dieback of grapevines (Lesuthu et al., 2019). It has since been reported causing dieback symptoms on *Cyclopia* (honeybush) species in a similar area of South Africa (Smit et al., 2021). While individual phylogenies based on the ITS, *tef* and *tub* gene regions were not sufficient to differentiate *D. nebulae* and *D. macadamiae*, differences in morphological description were documented (Lesuthu et al., 2019; Wrona et al., 2020). Analysis of additional gene regions of the isolates collected from macadamia fruits, as well as *D. nebulae* and *D. macadamiae* are therefore needed in order to determine if they should be regarded as different species or if the species should be reduced to synonymy.

Colletotrichum alienum, *C. fructicola*, *C. siamense* and *C. theobromicola* were successfully identified using multi-locus phylogenetic analyses for the first time in this study. Previous husk rot surveys in Australia, South Africa and Hawaii only referred to species as part of the *C. gloeosporioides* species complex (Akinsanmi & Drenth, 2017). The *Colletotrichum* species identified in this study are all known to cause anthracnose in various other crop systems, i.e. olives, pear, chilli, cotton, and citrus (Jayawardena et al., 2016; Kang et al., 2022; Moreira et al., 2021; Sharma & Shenoy, 2014; Wang et al., 2021). *C. siamense* is also reported to cause leaf spots in macadamia (Prasannath et al., 2020). *C. theobromicola*, the most frequently isolated in this study, is considered a serious pathogen of other crops including leaf disease of eucalypts and anthracnose of mango and olives (Dela Cueva et al., 2021; Lima et al., 2019; Solís et al., 2022).

The study presented strong phylogenetic evidence for the delineation of *Calonectria* sp. nov. 1 as gene regions had adequate support from both ML and BI analyses. Additional information, however, is required to determine the appropriateness of describing *Calonectria* sp. nov. 2 as a novel species. Aspects such as the addition of the *rpd2* gene region, morphology, mating type, and ecology will help to determine if these isolates are distinct enough not to be considered *Ca. pauciramosa.* For these reasons, neither of the species was described in this study. Identifying multiple *Calonectria* spp. causing very similar husk rot-like symptoms in South Africa was however unexpected as a single causal agent, *Calonectria pseudoreteaudii,*

was reported from two provinces in China (Jiang et al., 2019, 2020b). The study also confirmed the *Calonectria* species from the *Ca. candelabrum* species complex cause husk rot in South Africa whereas species from the *Ca. reteaudii* species complex are the causal agents of husk rot in China.

The distribution of *Colletotrichum* and *Diaporthe* species within a season and throughout the developmental stages of the macadamia fruit is a line of evidence of latent infection for both genera. *Diaporthe* in particular showed evidence for latency with the high isolation frequency in early and asymptomatic fruit stages. The presence of *Colletotrichum* species, initially at lower isolation frequencies from asymptomatic fruits throughout the developmental stages than matured fruits, is also typical of latent or opportunistic pathogens. The role of latent pathogen is not new in either *Diaporthe* or *Colletotrichum* as both genera have species known for latent infections in a number of other cropping systems including avocado, apple, pear, peaches, rubber trees, soybeans and citrus (Binyamini & Schiffmann-Nadel, 1972; Dai et al., 2019; de Silva et al., 2017; Du et al., 2021; Gomes et al., 2013; Sessa et al., 2018; Zhang et al., 1997). A recent publication also reported both *Diaporthe* and *Colletotrichum* isolated from macadamia seedlings in Australia (Sosso et al. 2021). The role of latency can therefore not be dismissed when considering the epidemiology of PHR and AHR husk rot.

The different *Calonectria* species identified in this study, from multiple locations, three years after the initial detection, suggest that CHR are already present in macadamia orchards and has likely been overlooked due to the similarity of symptoms to other husk rot types. The fact that *Calonectria* species can reside in soil and plant debris (Aiello et al., 2022; Crous, 2002; Li et al., 2022), combined with the pathogenicity trials that demonstrated that *Calonectria* species can produce husk rot-like symptoms without the aid of wounding, a prerequisite for most husk rot causal agents, suggests that a different management strategy will need to be undertaken in order to prevent potential economic damage to the industry. Future work focusing on the ecology and epidemiology of the CHR disease in-field conditions is therefore needed.

The different cultivars included in the pathogenicity assays for *Diaporthe* and *Colletotrichum* revealed variation in susceptibility. Nelmak 2 and HAES 695 (Beaumont) had similar levels of susceptibility to *Diaporthe* and *Colletotrichum* isolates whilst A4 showed a significant tolerance to both. Nelmak 2 is considered a very susceptible cultivar due to a tendency for maturing fruits to dehisce along the suture of the husk. The tolerance observed in A4 may be due to cultivar specific variations in husk thickness, biochemistry and/or physiology (Bolling et al., 2011; O'Connor et al., 2018). The ratio of the husk to shell to kernel is one of many traits not yet studied in macadamia that may affect the susceptibility of fruits to disease, similar to husk hardness that impacts pest damage (Hardner et al., 2009; O'Connor et al., 2018). Future breeding considering traits such as these may aid in generating cultivars with higher tolerance to fungal husk diseases as was observed in cultivar A4. Future studies should however, also include *Calonectria* species in cultivar screening assays.

Temperature growth studies demonstrated that the *Colletotrichum* isolates had a greater affinity for higher temperatures than did the *Diaporthe* isolates. Temperatures around January and February are generally higher than in November in the Mpumalanga province (Mpumalanga, South Africa Climate2020; https://tcktcktck.org/south-africa/mpumalanga) which may in part explain the increase in isolation frequency of *Colletotrichum* species. However, for *Calonectria*, 35 °C proved a lethal temperature after two weeks. The difference in response to temperature between *Diaporthe, Colletotrichum* and *Calonectria* may therefore improve our ability to predict conditions in which husk rot is more likely to develop and perhaps even when it is likely to spread (Miles et al., 2009, 2010a, 2010b). This can also aid in determining which climatic zones are at greater risk for each of the different husk rot diseases. The role of other environmental factors such as relative humidity and a better understanding of the lifecycle of the species involve are however also required to inform efficient and targeted management of husk rot.

This work has added to the body of knowledge around the pathogens causing husk rot, the potential lifestyles of these fungi on macadamia and the trends of pathogen dominance both seasonally and throughout development. Information such as the temperatures ranges for mycelial growth of the husk rot pathogens will aid in predicting risk periods and thus establishing more effective interventions.

Information gaps for husk rot include sources of inoculum in field, timing and method of infection and spread, and potential antagonistic or mutualistic interactions between the pathogens, especially *Diaporthe* and *Colletotrichum*, as both were often obtained from the same lesion in this study. This will create a solid information base for understanding and controlling husk rot in all growing regions of South Africa.

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Authors contribution Conceptualization: All authors. Methodology, analysis, and data curation: Dee Twiddy, Arista Fouché, Gerda Fourie. Funding acquisition: Gerda Fourie. Writing – original draft: Dee Twiddy. Writing – review & editing: All authors.

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Declarations

Statements and Declarations Olufemi A. Akinsanmi is editor of the European Journal of Plant Pathology. The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; and in the writing of the manuscript.

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