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ORIGINAL RESEARCH ARTICLE

Botryosphaeriaceae species associated with branch dieback and decline of macadamia trees in South Africa

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Abstract

Botryosphaeriaceae species are important latent pathogens causing diseases on trees utilized in forestry and agriculture. In recent years, there has been an increase in the incidence and severity of branch dieback and decline on macadamia trees in South Africa, and species of *Botryosphaeriaceae* have been considered as a possible cause. Although botryosphaeria dieback has been well-studied in Australia, there is little information regarding these fungi on Macadamia in South Africa. The aims of this study were consequently to (i) identify species of *Botryosphaeriaceae* from Macadamia branches from main production regions in South Africa, (ii) compare the diversity of species between symptomatic and asymptomatic branches, as well as between different growing regions, (iii) and to consider their relative importance in causing dieback. Eight species and three putative hybrids of the *Botryosphaeriaceae* were identified based on a phylogenetic comparison of sequence data from the ITS rDNA, *tub2*, *tef-1α* and *rpb2* loci. These included an unidentified *Diplodia* sp., and *Lasiodiplodia* sp., as well as *L. gilanensis, L. theobromae, L. pseudotheobromae, Neofusicoccum kwambonambiense, N. luteum, N. parvum* and three hybrid species. The unidentified species of *Diplodia., Lasiodiplodia* sp., *L. gilanensis,* and *N. kwambonambiense* are reported for the first time on Macadamia in South Africa. All species showed a potential to cause branch dieback symptoms, with species of *Neofusicoccum* identified as the most aggressive species. This study revealed a high level of diversity of *Botryosphaeriaceae* species and illustrates their potential as causal agents of dieback on Macadamia in South Africa.

Keywords Fungal endophyte · Species diversity · Pathogen · Aggressiveness

Introduction

Macadamia is a nut-bearing tree belonging to the family *Proteaceae*. It is indigenous to southeastern Australia, but is planted commercially in several tropical and subtropical countries of Africa, Asia, as well as North and South America in addition to Australia (Cann 1965; McConachie 1980; McHargue 1996). The Macadamia industry has expanded rapidly in recent years and today large areas in various countries are under cultivation. South Africa is currently the leading producer of Macadamia nuts, followed by Australia (SAMAC 2022). The increase in Macadamia production in the last few years is linked to the fact that it is the highest priced processed nut on the world market. Furthermore, Macadamia nuts are also highly sought after for their rich flavor, texture, and health benefits (Macfarlane and Harris 1981).

Macadamia trees, both in their native and introduced ranges, are commonly affected by various fungal pathogens that have resulted in significant yield losses affecting the productivity of the Macadamia industry (Akinsanmi et al. 2017; Drenth et al. 2009; Hunter and Kunimoto 1973; Ko and Kunimoto 1994; Wrona et al. 2020). Notable diseases include stem canker caused by *Phytophthora* species (Jeff-Ego et al. 2020), husk spot caused by *Pseudocercospora macadamiae* (Beilharz et al. 2003), dry flower disease caused by *Pestalotiopsis macadamiae* and *Neopestalotiopsis macadamiae* (Akinsanmi et al. 2017), phomopsis husk rot caused by *Diaporthe* species and botryosphaeria dieback caused by *Botryosphaeriaceae* species (Jeff-Ego and Akinsanmi 2019; Wrona et al. 2020).

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Branch dieback is one of the most commonly reported symptoms on woody crop plants (Jurskis 2005; Landsberg and Wylie 1988). It can be caused by multiple biotic factors including pests and diseases, as well as environmental stressors such as drought and salt damage (Jurskis 2005; Landsberg and Wylie 1988). *Phytophthora* and *Botryosphaeriaceae* species are known to cause branch dieback on Macadamia (Jeff-Ego and Akinsanmi 2019; Jeff-Ego et al. 2020). Branch dieback symptoms on Macadamia caused by *Phytophthora* include leaf chlorosis and loss of foliage (Jeff-Ego et al. 2020), while botryosphaeria branch dieback is characterized by browning of leaves that remain attached to trees accompanied by a wedge-shaped discoloration in cross-sections of the affected wood (Jeff-Ego and Akinsanmi 2019).

The *Botryosphaeriaceae* is a diverse family of fungi with a cosmopolitan distribution. Species are considered to be class III endophytes implying that they have the potential to become latent or opportunistic pathogens (Sakalidis et al. 2011; Mehl et al. 2013). As pathogens they often cause serious dieback and decline on a wide range of hosts (Slippers and Wingfield 2007; Mehl et al. 2013; Jami et al. 2014; Moral et al. 2019). Another notable aspect of the ecology of this group of fungi is their lack of host specificity, thus enabling them to colonize and cause disease on diverse native and introduced plant hosts in a particular region (Slippers and Wingfield 2007; Pérez et al. 2008; Mehl et al. 2013; Jami et al. 2014). Due to the endophytic nature of these fungi, they are easily introduced into new areas, unnoticed either on seeds or cuttings, later infecting other trees (Slippers and Wingfield 2007; Mehl et al. 2013; Marsberg et al. 2017).

The incidence and severity of Botryosphaeria dieback disease on Macadamia has increased significantly in Australia over the last 15 years (Jeff-ego and Akinsanmi 2019; Mohankumar et al. 2022). In total, thirteen *Botryosphaeriaceae* species have been associated with the branch dieback disease in Australia namely, *Botryosphaeria dothidea, Lasiodiplodia jatrophicola, L. iraniensis, L. pseudotheobromae, L. theobromae, Neofusicoccum australe, N. luteum, N. mangroviorum, N. parvum,* and four undescribed *Lasiodiplodia* species (Jeff-Ego and Akinsanmi 2019; Mohankumar et al. 2022). While these fungi are not as virulent as some primary pathogens, the dieback and canker diseases caused by *Botryosphaeriaceae* on Macadamia are amongst the most common and, under some conditions, the most serious problems affecting these trees.

In recent years, there have also been increasing reports of branch dieback and decline of Macadamia trees in South Africa. While considerable work has been done to identify species of *Botryosphaeriaceae* and their association with branch dieback in Australia, these fungi have hardly been studied on this host in South Africa. Only *Botryosphaeria ribis* has been reported causing disease on Macadamia in South Africa (Herbert and Grech 1985). The aim of this study was therefore to characterize species of *Botryosphaeriaceae* from Macadamia in South Africa and to consider their pathogenicity on this host.

Materials and methods

Disease symptoms, sampling, and fungal isolation

Samples were collected from the KwaZulu-Natal, Limpopo and Mpumalanga growing regions of South Africa in 2018. Samples were collected on two farms per region and at two sites per farm to capture cultivar diversity. At each farm, twenty asymptomatic branches were collected from 20 individual trees using random sampling. A total of 40 asymptomatic samples were therefore collected per growing region, with the number of diseased samples collected being dependent on availability. Symptoms observed in the field included general branch and shoot dieback, internal wood discoloration, and tree decline. The collected samples were placed in brown paper bags and processed the same day. To increase the number of samples obtained from diseased material, additional branch dieback samples received via the Forestry and Agricultural Biotechnology Institute (FABI) disease diagnostic clinic, University of Pretoria, were also included.

Branches were surface disinfested in 10% hydrogen peroxide for 1 min, in 70% alcohol for 1 min, and then rinsed in sterile water and dried on a paper towel. The branches were cut in half to expose the pith. For symptomatic samples, branches were inspected for evidence of discoloration in the pith and sections were taken from the edge of discoloured (necrotic) tissue. Four sterilized tissue sections of approximately 3–5 mm^2 from both symptomatic and asymptomatic branches were then placed onto the 2% malt extract agar (Biolab, MEA; 20 g malt extract, 20 g agar/1000 ml distilled water). The isolation plates were incubated at 25 °C for seven days. Single hyphal tips of isolates displaying characteristics of *Botryosphaeriaceae* (white to grey mycelium with aerial hyphae) were transferred to 2% MEA plates to obtain pure cultures. The pure cultures were subsequently deposited and are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria.

DNA extraction, PCR amplification and sequencing

DNA was extracted from fungal mycelium of 7-day-old cultures using the PrepMan ® Ultra kit (Thermo fisher Scientific) extraction method. Mycelium was scraped from the edges of colonies for each isolate using a sterile needle tip and transferred into a sterile 1.5-ml tube containing

50 µl of the PrepMan Ultra preparation reagent. Mycelial suspensions were shaken in a vortex mixer for 30 s and incubated at 95 °C in a heating block for 10 min. The tubes were centrifuged for 2 min and 50 µl of the supernatant was transferred to a new sterile 1.5-ml tube and stored at -20 °C until used for Polymerase Chain Reaction (PCR) reactions. DNA concentrations were determined using a ND-1000 spectrophotometer V3.7.1 (Thermo Fisher Scientific, USA) and diluted with Sabax water to 50 ng/µl.

The Internal Transcribed Spacer (ITS) gene region of the rRNA operon was amplified using the primers ITS1/ITS4 (White et al. 1990) for initial identification and placement of isolates in the genera of the *Botryosphaeriaceae*. Thereafter, additional gene regions were amplified for a subset of samples chosen to represent the various genera collected at different geographical origins, farm sites and cultivars (Online Resource Table 1). The Translation Elongation Factor (*tef-1α*) gene region was amplified using the primers EF1-728F/EF1-986R (Carbone and Kohn 1999), the β-tubulin (*tub*2) gene region using BT-2a/BT-2b (Glass and Donaldson 1995), and the RNA polymerase II subunit (*rpb*2) gene was amplified using the primers rpb2-LasF/rpb2-LasR for *Lasiodiplodia* (Cruywagen et al. 2017) and *rpb*2 bot6F/ RPB-2bot7R for *Neofusicoccum* (Pavlic et al. 2009; Sakalidis et al. 2011).

The total volume of the PCR mixture was 25 µL which consisted of 100–200 ng of genomic DNA, 5 µl of 5 mM MyTaq™ reaction buffer containing $MgCl_2$ and dNTPs (Bioline South Africa), 0.5 µl of MyTaq™ DNA polymerase, 0.5 µl of 10 mM primer for each gene region used, and 17.5 µl of ddH_2O (Adcock Ingram, Bryanston, South Africa). PCR was conducted in a thermal cycler (Bio-Rad, BioRad Laboratories Inc., Hercules, CA, USA) with the following cycles: initial denaturation at 95 °C for 4 min followed by 35 cycles of denaturation at 95 °C for 35 s; annealing at 55 °C (ITS, *tef1-α*, *tub*2), 56 °C (*rpb*2), for 1 min and extension at 72 °C for 90 s; followed by final extension for 10 min at 72 °C. The PCR amplification products were separated by electrophoresis in 1% agarose gels and run at 100 V and 400 mA for 40 min in 1.0 × Tris-borate-EDTA (TBE) buffer. PCR product sizes were estimated using a 100 bp marker and visualized using Bio-Rad Molecular Imager® Gel Doc™ XR System. This was followed by a PCR product clean up reaction using ExoSAP-IT™ (Applied Biosystems, Foster city, CA) according to the manufacturer's instructions.

BigDye Terminator v3.1 Cycle Sequencing Kit was used to sequence PCR products in both directions. The thermal cycler was programmed as follows: initial denaturation at 96 °C for 2 min, followed by 25 cycles at 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. The samples were sequenced using an ABI 3100 Automated Capillary DNA Sequencer (Applied Biosystems, USA) at the Bioinformatics Sequencing facility, University of Pretoria.

Phylogenetic analyses

Forward and reverse sequences were checked for accuracy and manually edited using CLC BioWorkbench v.5. Consensus sequences were generated by aligning the forward and reverse sequences. Sequences were submitted to GenBank (Online Resource Table 1). The generated ITS sequences were then subjected to Basic Local Alignment Search Tool (BLAST) searches against the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) to group the isolates into different genera. Thereafter, ITS, *tub2*, *tef-1α* and *rpb*2 sequences of each genus were compiled into a dataset that included known species. The reference sequences and type strains were obtained from papers published by Cruywagen et al. (2017), Pavlic et al. (2009), Phillips et al. (2013) and Zhang et al. (2021). Additional four novel species recently described by Mohankumar et al. (2022) were also included. The sequence dataset was then aligned with Multiple Alignment using Fast Fourier Transform (MAFFT) version 7 (https://mafft.cbrc.jp/alignment/software).

Phylogenetic analyses of single genes and the concatenated sequence data based on maximum likelihood (ML) were performed by using Randomized Axelerated Maximum Likelihood (RaxML) version 8 (Stamatakis 2014) and viewed in Mega 7.0. Individual trees were generated, and the best substitution models were determined for each dataset with Modeltest v.2.1.3 using the Akaike Information Criterion (AIC). The best-fit evolution model for ITS and *tef1-α* was GTR + G (Stamatakis and Alachiotis 2010) whereas HKY + G was the best model for *tub2* and *rpb2* (Hasegawa et al. 1985). For ML analyses, a bootstrap value of 1000 was set to determine the robustness of the trees. Bayesian inference, based on a Markov Chain Monte Carlo (MCMC) approach, was also performed in MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003). Datasets for each of the gene regions sequenced were analysed separately and combined to investigate the congruency of the phylogenetic clades obtained. All the phylogenetic trees generated in this study are rooted to *Botryosphaeria dothidea*. Based on the results of ML, species recognition was determined according to the genealogical species concordance.

Pathogenicity trials

Species of *Botryosphaeriaceae* that were isolated more than once were used in pathogenicity tests. A single isolate representing each of these species was randomly selected for the pathogenicity trials under glasshouse conditions (Online Resource Table 1). The isolates were grown on 2% MEA at

Table 1 Botryosphaeriaceae isolates used in the phylogenetic analysis and pathogenicity trials in this study

CMW	Species	Tissue[3]	Province	Farm	Cultivar	Date	GenBank Accession number			
							ITS	*tub2*	*tef-1α*	*rpb2*
57,110	*Diplodia* sp.	H	Limpopo	1	Beaumont	2018	OR671504	OR770372	OR761223	-
57,111	*Diplodia* sp.	H	Limpopo	1	Beaumont	2018	OR671505	OR761373	OR761224	-
57,172	*L. gilanensis*	D	KwaZulu-Natal	2	Beaumont	2018	OR659658	OR770462	OR772784	OR772795
57,188	*L. gilanensis*	D	KwaZulu-Natal	2	Beaumont	2018	OR659659	OR770463	OR772783	OR772796
57,182	*L. gilanensis*	D	KwaZulu-Natal	2	Beaumont	2018	OR659660	OR770464	OR772782	OR772797
57,180	*L. gilanensis*	D	KwaZulu-Natal	2	Beaumont	2018	OR659661	OR770465	OR772781	OR772798
57,181	*L. gilanensis*	H	Limpopo	3	Beaumont	2018	OR659662	OR770466	OR772785	OR772799
57,163	*L. gilanensis*	H	Limpopo	3	Beaumont	2018	OR659663	OR770467	OR772786	OR772800
57,164	*L. gilanensis*	H	Limpopo	3	Beaumont	2018	OR659664	OR770468	OR772787	OR772801
57,166	*L. gilanensis*	H	Limpopo	3	Beaumont	2018	OR659665	OR770469	OR772788	OR772802
57,167	*L. gilanensis*	H	Limpopo	1	Beaumont	2018	OR659666	OR770470	OR772789	OR772803
57,184	*Lasiodiplodia* sp.	D	Mpumalanga	1	Mixed int	2018	OR659667	OR770481	OR772900	OR773001
57,185	*Lasiodiplodia* sp.	D	Mpumalanga	1	Mixed int	2018	OR659677	OR770482	OR773670	OR773002
57,195	*Lasiodiplodia* sp.	H	Mpumalanga	4	Nelmak D	2018	OR659678	OR770483	OR773671	OR773003
57,196	*Lasiodiplodia* sp.	H	Mpumalanga	5	816	2018	OR659679	OR770484	OR773672	OR773004
57,187	*Lasiodiplodia* sp.	H	KwaZulu-Natal	2	788	2018	OR659680	OR770486	OR773673	OR773005
57,191	*Lasiodiplodia* sp.	H	KwaZulu-Natal	2	788	2018	OR659681	OR770487	OR773674	OR773006
57,192	*Lasiodiplodia* sp.	H	Mpumalanga	4	Nelmak D	2018	OR659683	OR770488	OR773675	OR773007
57,201	*Lasiodiplodia* sp.	H	Mpumalanga	4	344	2018	OR659682	OR770489	OR773676	OR773008
57,202	*Lasiodiplodia* sp.	H	Mpumalanga	5	816	2018	OR659676	OR770470	OR773677	OR773009
57,206	*Lasiodiplodia* sp.	H	KwaZulu-Natal	2	Beaumont	2018	OR671508	OR770362	OR770678	OR770299
57,200	*Lasiodiplodia* sp.	H	Mpumalanga	1	788	2018	OR671509	OR770364	OR770679	OR770297
57,203	*Lasiodiplodia* sp.	H	KwaZulu-Natal	6	Nelmak D	2018	OR772782	OR770355	OR671549	OR671674
57,199	*Lasiodiplodia* sp.	H	KwaZulu-Natal	6	Nelmak D	2018	OR772783	OR770354	OR671547	OR671673
57,197	*Lasiodiplodia* sp.	H	KwaZulu-Natal	2	Beaumont	2018	OR772784	OR770353	OR671548	OR671672
57,204	*Lasiodiplodia* sp.	H	KwaZulu-Natal	2	788	2018	OR772786	OR770352	OR671546	OR671671
57,207	*Lasiodiplodia* sp.	H	Mpumalanga	4	Nelmak D	2018	OR772787	OR770351	OR671543	OR671670
57,209	*Lasiodiplodia* sp.	H	Mpumalanga	4	344	2018	OR772788	OR770350	OR671542	OR671669
57,309	*L. pseudotheobromae*	D	KwaZulu-Natal	2	Beaumont	2018	OR772796	OR680840	OR772794	OR772801
57,310	*L. pseudotheobromae*	D	KwaZulu-Natal	2	Beaumont	2019	OR772792	OR680841	OR770468	OR772802
57,302	*L. pseudotheobromae*	D	Limpopo	1	Mixed int	2018	OR772793	OR680842	OR649170	OR772803
57,321	*L. pseudotheobromae*	D	Limpopo	7	814	2018	OR772790	OR680844	OR649171	OR772804
57,319	*L. pseudotheobromae*	D	KwaZulu-Natal	2	Beaumont	2021	OR772794	OR680845	OR649172	OR772805
57,316	*L. pseudotheobromae*	D	KwaZulu-Natal	2	Beaumont	2020	OR772797	OR680846	OR649173	OR772806
57,327	*L. pseudotheobromae*	D	KwaZulu-Natal	2	Beaumont	2022	OR772798	OR680847	OR649174	OR772807
57,140	*L. pseudotheobromae*	D	Limpopo	7	814	2018	OR772799	OR680848	OR649182	OR772808
57,160	*L. pseudotheobromae*	D	Mpumalanga	8	Mixed int	2020	OR772800	OR680849	OR770468	OR772809
57,161	*L. pseudotheobromae*	D	Mpumalanga	8	Mixed int	2020	OR772801	OR680850	OR770441	OR772900
57,162	*L. pseudotheobromae*	D	Mpumalanga	9	Mixed int	2020	OR772802	OR680861	OR770442	OR680832
57,153	*L. pseudotheobromae*	D	KwaZulu-Natal	10	Mixed int	2019	OR772803	OR680862	OR770443	OR680833
57,154	*L. pseudotheobromae*	D	KwaZulu-Natal	10	Mixed int	2019	OR772804	OR680863	OR770444	OR680834
57,155	*L. pseudotheobromae*	D	KwaZulu-Natal	10	Mixed int	2019	OR772805	OR680864	OR770446	OR680832
57,156	*L. pseudotheobromae*	D	KwaZulu-Natal	10	Mixed int	2019	OR772806	OR680865	OR770447	OR680833
57,157	*L. pseudotheobromae*	D	KwaZulu-Natal	11	Beaunont	2019	OR772807	OR680866	OR770448	OR680815
57,158	*L. pseudotheobromae*	D	KwaZulu-Natal	11	Beaumont	2019	OR772808	OR680843	OR770449	OR680816
57,159	*L. pseudotheobromae*	D	KwaZulu-Natal	11	Beaumont	2019	OR772809	OR680842	OR772793	OR680817
57,170	*L. pseudotheobromae*	D	KwaZulu-Natal	11	Beaumont	2019	OR772900	OR680841	OR772792	OR680818
57,311	*L. pseudotheobromae*	H	Limpopo	12	Mixed int	2018	OR772901	OR680840	OR772791	OR680819
57,314	*L. pseudotheobromae*	H	Limpopo	12	Mixed int	2018	OR772902	OR680839	OR770490	OR680820

Table 1 (continued)

CMW	Species	Tissue[3]	Province	Farm	Cultivar	Date	GenBank Accession number			
57,306	*L. pseudotheobromae*	H	Limpopo	12	Mixed int	2018	OR772903	OR770371	OR770339	OR680821
57,304	*L. pseudotheobromae*	H	Limpopo	12	Mixed int	2018	OR659668	OR770370	OR770338	OR680822
57,308	*L. pseudotheobromae*	H	Limpopo	12	Mixed int	2018	OR659669	OR770369	OR770337	OR680823
57,307	*L. pseudotheobromae*	H	Limpopo	12	Mixed int	2018	OR659670	OR770368	OR770336	OR680824
57,303	*L. pseudotheobromae*	H	Limpopo	12	Mixed int	2018	OR659671	OR770367	OR770334	OR680825
57,301	*L. pseudotheobromae*	H	Limpopo	12	Mixed int	2018	OR659674	OR770366	OR770333	OR680826
57,300	*L. pseudotheobromae*	H	Mpumalanga	4	Nelmak D	2018	OR659675	OR770364	OR770332	OR680827
57,312	*L. pseudotheobromae*	H	Limpopo	12	Mixed int	2018	OR659672	OR770363	OR770331	OR680828
57,313	*L. pseudotheobromae*	H	Limpopo	12	Mixed int	2018	OR659673	OR770362	OR770330	OR680829
57,169	*L. pseudotheobromae*	H	Mpumalanga	4	Nelmak D	2018	OR659676	OR680843	OR770448	OR680830
57,152	*L. pseudotheobromae*	H	Limpopo	12	Mixed int	2018	OR659657	OR770469	OR770449	OR680831
57,138	*L. pseudotheobromae*	H	KwaZulu-Natal	13	788	2018	OR659667	OR770468	OR770470	OR680835
57,130	*L. theobromae*	D	Limpopo	1	Mixed int	2018	OR659651	OR770467	OR770481	OR680832
57,134	*L. theobromae*	D	Mpumalanga	4	344	2018	OR659652	OR770466	OR770482	OR680833
57,129	*L. theobromae*	D	Limpopo	7	Mixed int	2018	OR659653	OR770465	OR770483	OR680834
57,132	*L. theobromae*	H	Mpumalanga	4	Nelmak D	2018	OR659654	OR770464	OR770484	OR680836
57,131	*L. theobromae*	H	Mpumalanga	5	816	2018	OR770468	OR770463	OR770486	OR680840
57,128	*L. theobromae*	H	Limpopo	1	Mixed int	2018	OR659656	OR770462	OR770487	OR680830
57,136	*L. theobromae*	H	KwaZulu-Natal	2	Beaumont	2018	OR659668	OR770461	OR770488	OR680831
57,133	*L. theobromae*	H	Mpumalanga	4	Nelmak D	2018	OR659669	OR770460	OR770489	OR680835
57,135	*L. theobromae*	H	KwaZulu-Natal	2	Beaumont	2018	OR770468	OR770459	OR770468	OR680832
57,112	*N. kwambonambiense*	D	Limpopo	2	Beaumont	2018	OR649170	OR680821	OR770348	OR680815
57,114	*N. kwambonambiense*	D	Limpopo	2	Beaumont	2018	OR649171	OR680822	OR770347	OR680816
57,116	*N. kwambonambiense*	D	Limpopo	2	Beaumont	2018	OR649172	OR680823	OR770346	OR680817
57,118	*N. kwambonambiense*	D	Limpopo	14	Beaumont	2018	OR649173	OR680824	OR770345	OR680818
57,113	*N. kwambonambiense*	D	Limpopo	2	Beaumont	2018	OR649174	OR680821	OR770344	OR680819
57,115	*N. kwambonambiense*	D	Limpopo	1	Beaumont	2017	OR649182	OR680822	OR770343	OR680827
57,123	*N. kwambonambiense*	D	Limpopo	15	Beaumont	2020	OR649181	OR770330	OR770342	OR680826
57,120	*N. kwambonambiense*	H	Limpopo	14	Beaumont	2018	OR649175	OR770331	OR770341	OR680820
57,117	*N. kwambonambiense*	H	Limpopo	14	Beaumont	2018	OR649176	OR770332	OR770340	OR680821
57,121	*N. kwambonambiense*	H	KwaZulu-Natal	13	Beaumont	2018	OR649177	OR770334	OR770339	OR680822
57,119	*N. kwambonambiense*	H	Limpopo	14	Beaumont	2018	OR649178	OR770335	OR770338	OR680823
57,124	*N. kwambonambiense*	H	Limpopo	2	Mixed int	2018	OR649179	OR770328	OR770337	OR680824
57,122	*N. kwambonambiense*	H	Limpopo	2	Mixed int	2018	OR649180	OR770327	OR770336	OR680825
57,125	*N. luteum*	D	KwaZulu-Natal	16	Mixed int	2020	OR649183	OR770326	OR770468	OR680843
57,126	*N. luteum*	D	KwaZulu-Natal	16	Mixed int	2020	OR649184	OR770325	OR770469	
57,127	*N. luteum*	D	Mpumalanga	9	Mixed int	2020	OR649185	OR770324	OR770470	OR680844
57,140	*N. parvum*	D	Limpopo	18	Mixed int	2020	OR649168	OR770323	OR770481	OR680829
57,145	*N. parvum*	D	KwaZulu-Natal	16	Mixed int	2020	OR649167	OR770322	OR770482	OR680830
57,146	*N. parvum*	D	Limpopo	17	Beaumont	2019	OR649166	OR770321	OR770483	OR680831
57,149	*N. parvum*	D	Limpopo	17	Beaumont	2019	OR649162	OR770320	OR770484	OR680835
57,143	*N. parvum*	D	Limpopo	17	Beaumont	2019	OR649165	OR770319	OR770486	OR680832
57,144	*N. parvum*	D	Limpopo	17	Beaumont	2019	OR649164	OR770318	OR770487	OR680833
57,142	*N. parvum*	D	Limpopo	17	Beaumont	2019	OR649163	OR772801	OR770488	OR680834
57,150	*N. parvum*	H	Limpopo	2	Beaumont	2018	OR649161	OR772802	OR770489	OR680836
57,155	*N. parvum*	H	Limpopo	2	Mixed int	2018	OR649157	OR772803	OR770468	OR680840
57,152	*N. parvum*	H	Limpopo	2	Beaumont	2018	OR649160	OR772804	OR770469	OR680837
57,154	*N. parvum*	H	Limpopo	2	Mixed int	2018	OR649158	OR772805	OR770470	OR680839
57,158	*N. parvum*	H	Limpopo	2	Beaumont	2018	OR649159	OR772806	OR770481	OR680838
57,156	*N. parvum*	H	Limpopo	2	Beaumont	2018	OR649156	OR772807	OR770482	OR680841

Table 1 (continued)

CMW	Species	Tissue[3]	Province	Farm	Cultivar	Date	GenBank Accession number			
57,151	*N. parvum*	H	Limpopo	2	Beaumont	2018	OR649155	OR772808	OR770483	OR680842
57,141	*N. parvum*	H	Limpopo	2	Mixed int	2018	OR649169	OR772809	OR770484	OR680828

[1]ID numbers for isolates used in pathogenicity trials in this study are given in bold

[2]Isolates shown to hybridize are given in italics

[3]Isolates obtained from asymptomatic tissues = H, and from symptomatic tissues = D

[4]Mixed Int = Mixed integrifolia where the correct identification of the cultivar was not clear or unknown

room temperature for seven days prior to inoculation. One-year-old trees of the Beaumont cultivar, planted in black plastic bags, were left to acclimatize in the glasshouse for 3 weeks prior to inoculation. Glasshouse conditions were set with daily temperature fluctuations (approximately 25 °C Day/15 °C night), and a controlled photoperiod of 16/8 h (day/night).

Ten inoculations per isolate were performed at the base of the stem of each tree by firstly removing the bark tissue with a 5 mm cork borer to expose the cambium. Thereafter a 5 mm diameter mycelial plug, cut from the actively growing margin of seven-day-old culture, were placed mycelium surface down into the wounded area with control inoculations that were made using a clean MEA plug. The wounds and mycelial plugs were then sealed with Parafilm (Bemis®, USA) to prevent desiccation and contamination. The inoculated trees were randomly arranged in the glasshouse. Following inoculation, trees were watered every two days for the first 9 weeks. After the ninth week, trees were watered every fourth day. Measurements of lesions were taken when the first dieback symptoms were observed on the trees 12 weeks after inoculation. Data visualization and statistical analyses for all data were carried out in R software (RStudio Team, 2022). The Kruskal–Wallis test was used to identify the significant difference ($P < 0.05$) between species used in the pathogenicity trials in this study.

Re-isolations were made from two trees inoculated with each of the *Botryosphaeriaceae* isolates and the controls. This was done by cutting out a small piece of wood at the edge of the lesion and placing this on 2% MEA. DNA sequences of the *tef-1α* gene region were used to confirm the identity of the fungal isolates obtained.

Results

Fungal Isolates

A total number of 138 *Botryosphaeriaceae* isolates were obtained from symptomatic and asymptomatic branches. We therefore isolated 92 *Botryosphaeriaceae* isolates from 120 asymptomatic branches. Of the 46 isolates obtained from symptomatic tissues, 27 were obtained from disease clinic samples and 15 were from samples collected from the survey of the three regions. With regards to geographical location, 58 isolates were obtained from Limpopo, 33 isolates from Mpumalanga, and 47 isolates from KwaZulu-Natal. BLASTn results revealed that the isolates obtained belonged to three genera, namely *Diplodia* (two isolates) *Lasiodiplodia* (88 isolates) and *Neofusicoccum* (48 isolates).

Identity of isolates

In the phylogenetic analysis the two *Diplodia* isolates grouped in a clade with *D. alatafructa, D. pseudoseriata* and *D. insularis* (Fig. 1). The two isolates were identical to the type strains of *D. pseudoseriata* (EU080927) and *D. alatafructa* (FJ888460) in the ITS dataset (Online Resource 1a). However, the two isolates formed a sub-clade within a larger clade in both the *tub*2 (Online Resource 1b) the *tef-1α* (Online Resource 1c) phylogenies with a strong bootstrap support. In the combined phylogeny, the two isolates grouped in a clade with *D. alatafructa, D. pseidoseriata* and *D. insularis*, in agreement to the grouping observed in the ITS phylogeny (Fig. 1). The two isolates in the present study differed from *D. pseudoseriata* with three and two nucleotides on *tef-1α* and *tub*2, respectively, and consequently could not be assigned to a known species and are treated as *Diplodia* sp.

The phylogenetic analyses of ITS, *tub2*, *tef-1α*, *rpb2* and combined sequence datasets of the 31 representative isolates of *Neofusicoccum* resulted in trees with similar topologies (Fig. 2, Online resource 2). The phylogenies grouped the isolates into three clades representing *N. parvum* ($n = 15$), *N. kwambonambiense* ($n = 13$), and a clade containing *N. luteum* and *N. mangroviorum* ($n = 3$) (Fig. 2). Based on our phylogenetic analysis, the latter two species could not be confidently separated from one another, which agrees with Zhang et al. (2021).

A total of 60 *Lasiodiplodia* representative isolates were selected for further analysis, however only a few representatives are shown in the individual and combined phylogenies. Phylogenetic analyses of the representative isolates based on both the ITS and *tub2* datasets lacked

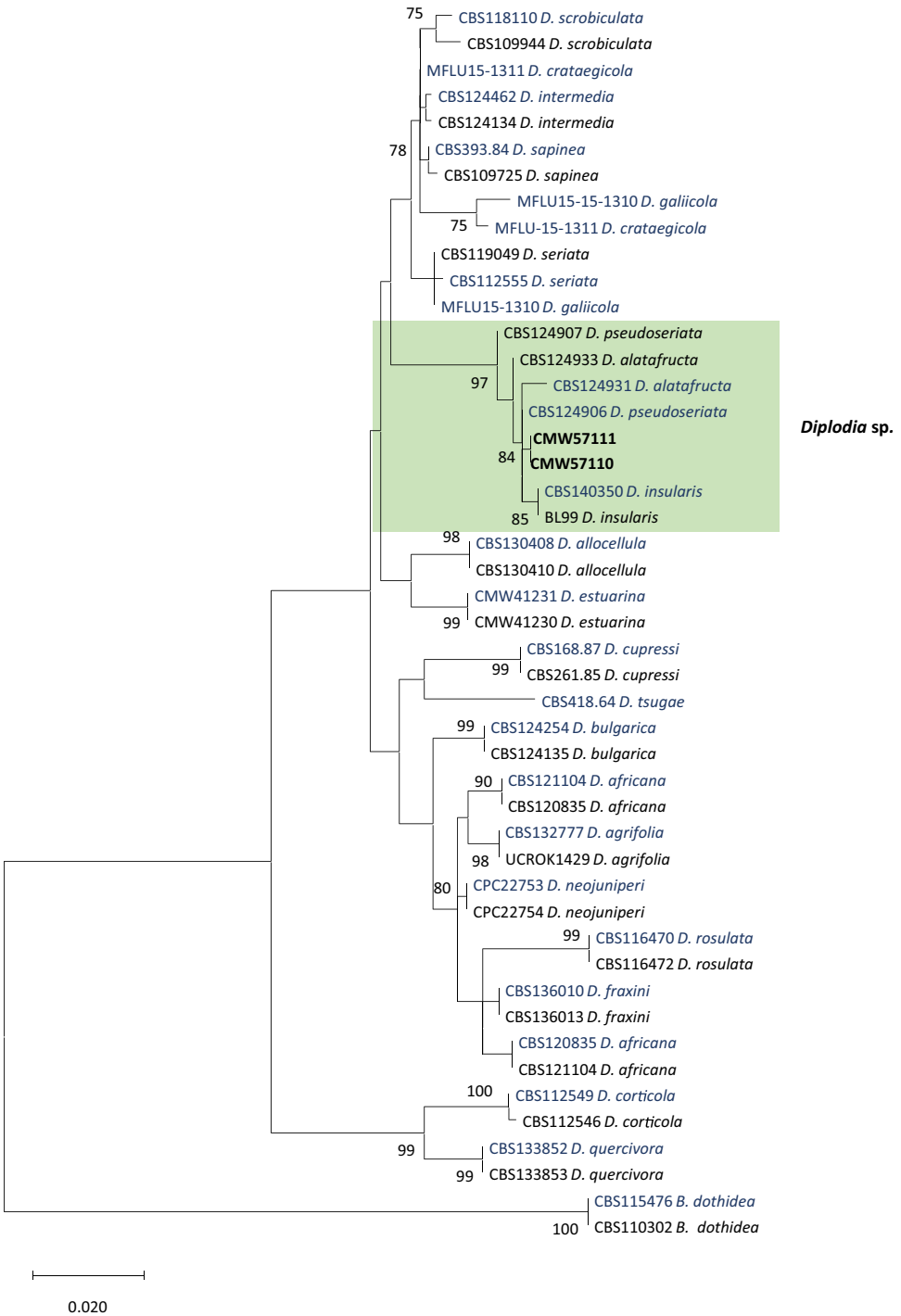

Fig. 1 Maximum Likelihood (ML) tree of the genus *Diplodia* produced with from a combined ITS, *tub2,* and *tef-1α* sequences of *Diplodia*. Bootstrap values above 70% are given at the nodes. Isolates sequenced in this study appear in bold and type species are in blue. The tree was rooted to *Botryosphaeria dothidea* (CBS115476 and CBS110302)

phylogenetic resolution to differentiate between all the known *Lasiodiplodia* species (Online Resources 3a, b). Eight of the isolates grouped closely but separate from *L. gonubiensis*, while the remaining *Lasiodiplodia* isolates grouped within a larger clade representing the *L. theobromae* species complex (Online Resources 3a, b). The *tef-1α* and *rbp2* phylogenies (Online Resources 3c, d) had similar topologies and could distinguish between most *Lasiodiplodia* species in this study. In both phylogenies, nine isolates grouped with *L. gilanensis* and *L. missouriana* (syn. *L. gilanensis*; Zhang et al. 2021) and another eight isolates grouped with *L. pseudotheobromae.* The combined dataset of the four gene regions (Fig. 3) confirmed the grouping of isolates from this study in *L. pseudotheobromae, L. theobromae, L. gilanensis* and *Lasiodiplodia* sp.

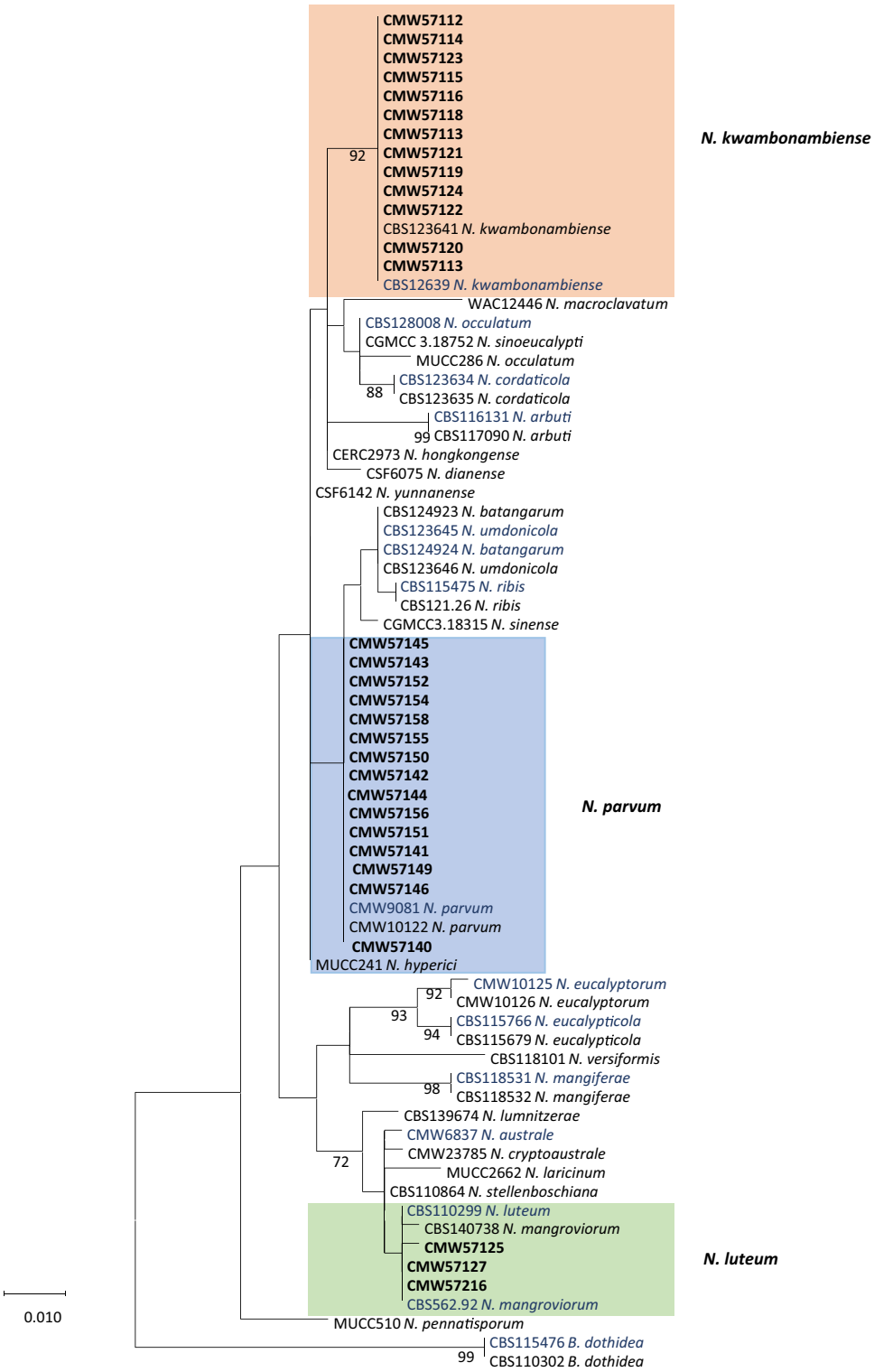

Fig. 2 Maximum Likelihood (ML) tree of *Neofusicoccum* produced with a combined sequence data of ITS, *tub2*, *rpb2* and *tef-1α*. Bootstrap values above 70% are given at the nodes. Isolates sequenced in this study appear in bold and type species are in blue. The tree was rooted to *Botryosphaeria dothidea* (CBS115476 and CBS110302)

Three of the isolates obtained in this study (CMW57135, CMW57129 and CMW57133) displayed incongruency between different gene regions and might represent hybrids. In ITS and *tub2* (Online Resources 3a, b), all three isolates grouped within the *L. theobromae* complex. However, for *tef-1α* (Online Resource 3c), CMW57133 grouped with *L. pseudotheobromae* and with *L. theobromae* in the *rpb2* dataset. Isolate CMW57129 grouped separately, but close to *L. theobromae* in *tef-1α*, but grouped with *L. mahajangana* (syn. *L. exigua*) in the *rpb2* analysis (Online Resource 3d)

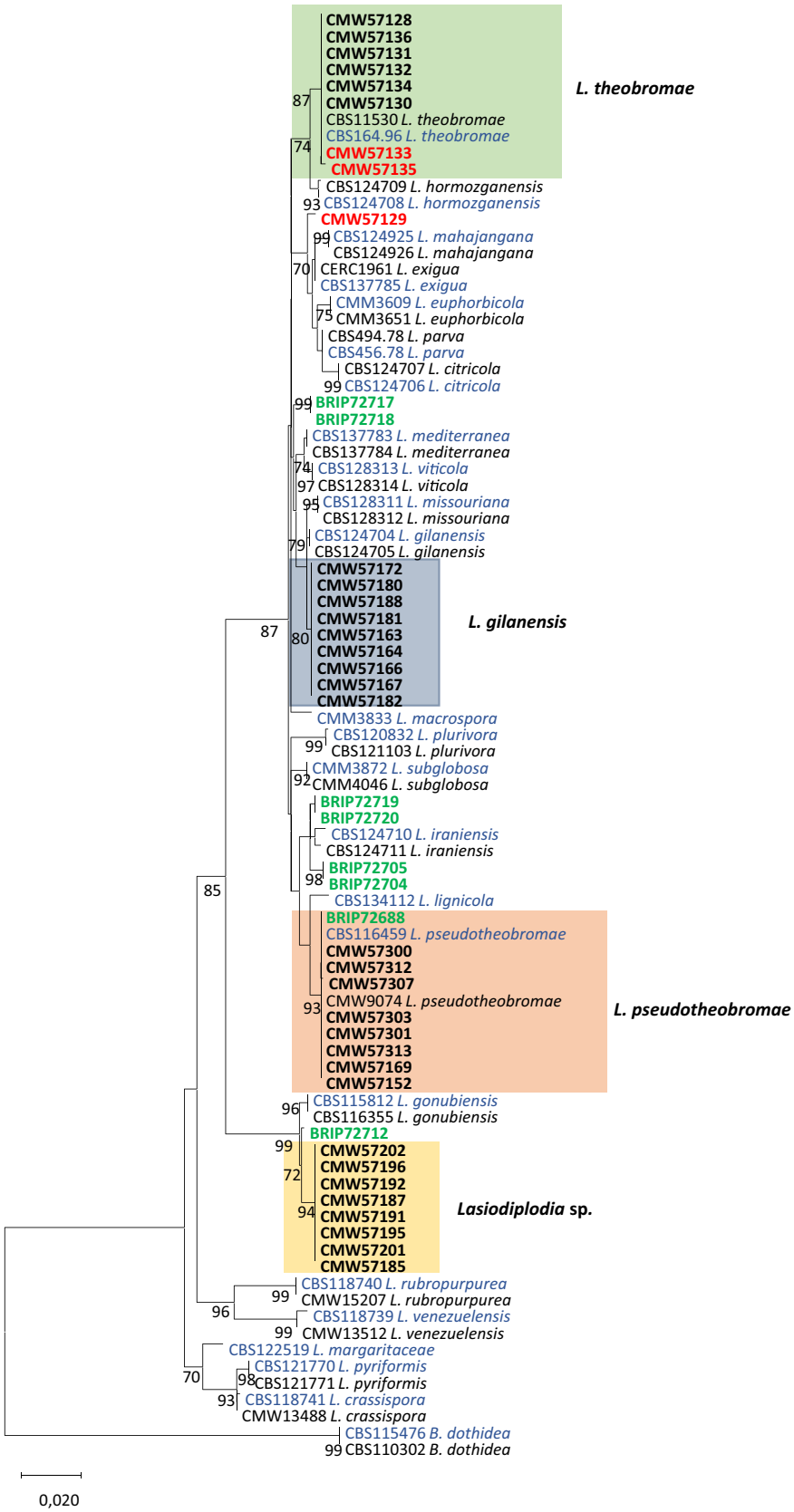

Fig. 3 Maximum Likelihood (ML) tree of *Lasiodiplodia* produced with a combined sequence data of *ITS, tub2, rpb2 and tef-1α*. Bootstrap values above 70% are given at the nodes. Isolates sequenced in this study appear in bold and type species are in blue. Potential hybrid isolates are given in red and isolates from Australia are in green. The tree was rooted to *Botryosphaeria dothidea* (CBS115476 and CBS110302)

and in the combined dataset (Fig. 3). Isolate CMW57135 grouped with *L. theobromae* in all individual datasets other than for *tef-1α* where it grouped close to both *L. pseudotheobromae* and *L. lignicola.* Based on the decision tree provided by Cruywagen et al. (2017), all three isolates were thus treated as hybrids because they move between non-sister species in phylogenetic analyses.

Species distribution per region

Botryosphaeriaceae species were recovered from all three major Macadamia growing regions in South Africa, however the species diversity and distribution differed between regions. Seven species was found in KwaZulu-Natal, with six species present in Limpopo and four in Mpumalanga (Fig. 4). One putative hybrid species was also detected in each growing region. *Lasiodiplodia pseudotheobromae* was the most frequently isolated species ($n = 33$), followed by *Lasiodiplodia theobromae* ($n = 14$) from all three Macadamia production regions. *Lasiodiplodia* sp. ($n = 29$) was isolated in relative high numbers from Mpumalanga and KwaZulu-Natal while *N. parvum* ($n = 24$) was dominant in Limpopo with only one isolate obtained from KwaZulu-Natal. *Neofusicoccum kwambonambiense* ($n = 21$), *N. luteum* ($n = 3$) and *L. gilanesis* ($n = 9$) were obtained from both KwaZulu-Natal and Limpopo. The *Diplodia* sp. were present in very low numbers and were only isolated from a single orchard in Limpopo (Fig. 4).

Isolates from asymptomatic versus symptomatic trees

A total of 92 isolates isolates were obtained from 120 asymptomatic tissues, while all 46 symptomatic tissues yielded 46 isolates. Six of the eight species (*N. kwambonambiense N. parvum, Lasiodiplodia* sp. *L. pseudotheobromae, L. gilanensis,* and *L. theobromae*) identified in this study were present on both tissue types (Fig. 5). *Lasiodiplodia pseudotheobromae* was the most common and dominant species in both tissue types. However, it was isolated in higher numbers from diseased tissues ($n = 19$) compared to healthy tissues ($n = 14$). *Diplodia* sp. was obtained from only asymptomatic tissues but was isolated in only low numbers ($n = 2$) and from a single orchard. *Neofusicoccum luteum* was also represented in low numbers ($n = 3$) and was isolated only from diseased samples submitted to the diagnostic clinic. Of the three *Lasiodiplodia* hybrids, two were isolated from asymptomatic tissues (CMW57133 and CMW57135) while CMW 57129 was isolated from a symptomatic tissue.

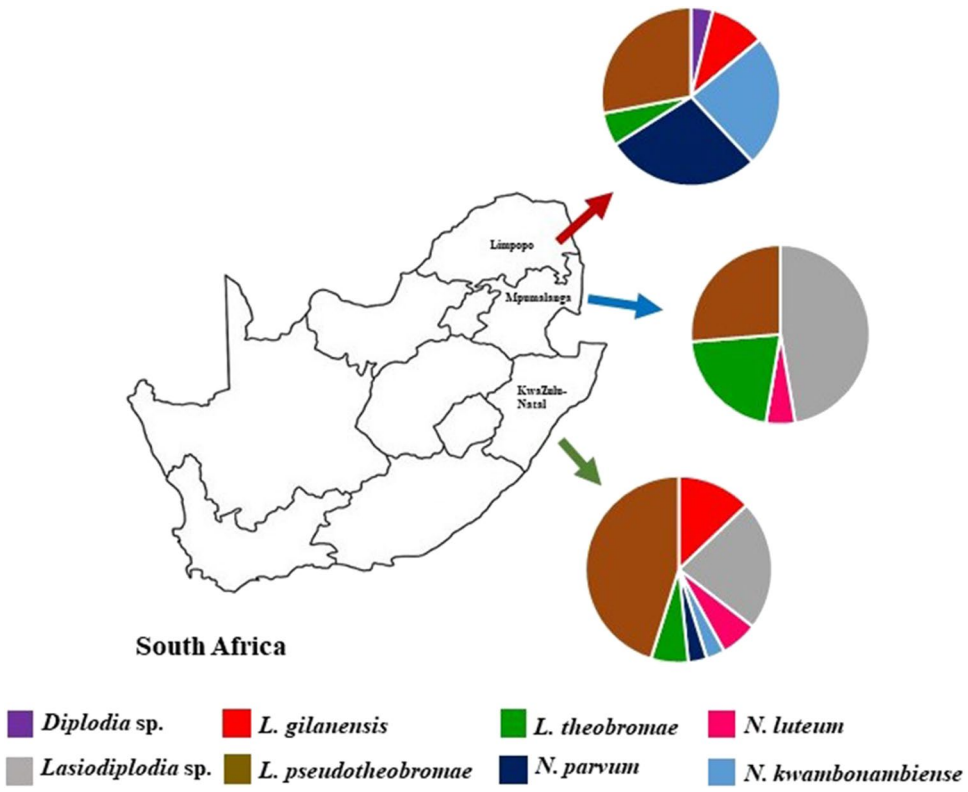

Fig. 4 *Botryosphaeriaceae* species diversity and distribution on Macadamia across three the major growing regions in South Africa

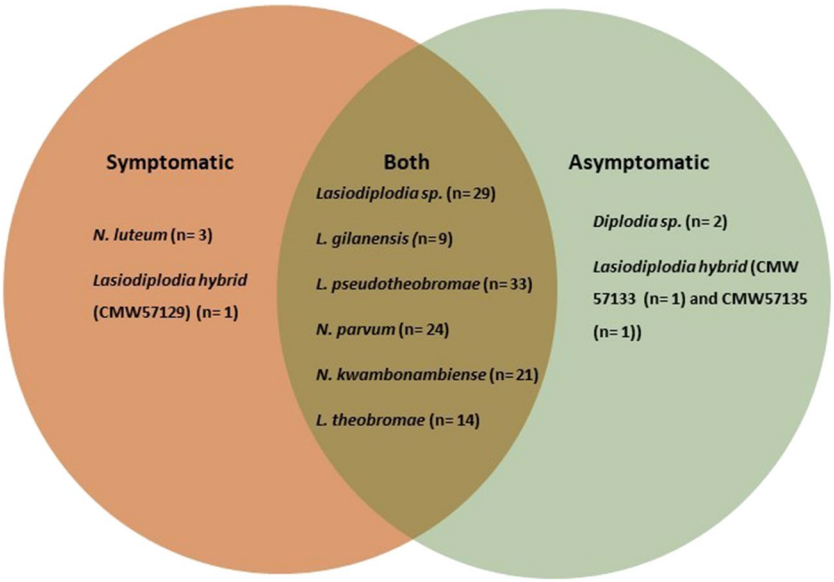

Fig. 5 Venn diagram showing species overlap and diversity of species obtained from both asymptomatic and symptomatic tissues

Pathogenicity trials

For the first nine weeks, the inoculated plants showed no visible leaf symptoms and no stem discoloration (necrotic lesion) (Fig. 6a). Lesions and branch dieback symptoms were however observed 12 weeks post inoculation (Fig. 6b, c, d), three weeks after watering of plants was reduced. All the *Botryosphaeriaceae* isolates tested in this study produced lesions after wounding and are therefore considered pathogenic to Macadamia. Significant ($P < 0.001$) differences in aggressiveness were observed among the species. *Neofusicoccum luteum*, *N. kwambonambiense* and *Lasiodiplodia* sp. produced the largest lesions and their lesions differed significantly from those of the controls (Fig. 7). *Lasiodiploidia gilanensis, L. pseudotheobromae* and *L. theobromae* produced the smallest lesions of all tested isolates.

Discussion

This study represents the first comprehensive investigation of the diversity, distribution, and the pathogenicity of *Botryosphaeriaceae* species associated with macadamia branches in South Africa. Isolates obtained from different growing regions were analysed, resulting in the identification of eight species and three putative hybrids residing in *Diplodia*, *Neofusicoccum* and *Lasiodiplodia*. These included a *Diplodia* sp., three *Neofusicoccum* species and four *Lasiodiplodia* species. In addition, three putative *Lasiodiplodia* hybrids were also identified. The pathogenicity trials demonstrated that all *Botryosphaeriaceae* species linked to Macadamia in South Africa can induce vascular discoloration and/or dieback in Macadamia seedlings, although symptoms only appeared after a significant reduction in irrigation.

This study revealed a high diversity of *Botryosphaeriaceae* species on Macadamia in South Africa. This in agreement with previous studies from other woody hosts (McDonald and Eskalen 2011; Jami et al. 2013; Carlucci et al. 2015; Scarlett et al. 2019; Hilário et al. 2020). A total of thirteen species were also obtained from surveys conducted on Macadamia in Australia (Jeff-Ego and Akinsanmi 2019; Mohankumar et al. 2022). The results obtained from this study are therefore in agreement with the surveys from Australia that revealed a high diversity of species present on this host in both its native and non-native range. However, in terms of species overlap, only *L. pseudotheobromae, L. theobromae, N. parvum* and *N. luteum* (syn. *N. mangroviorum*) were isolated from Macadamia in both countries. These shared species are known to have a cosmopolitan distribution associated with various hosts including wood, fruit and nuts crops (Slippers and Wingfield 2007; Trouillas et al. 2010; Hui-Fang et al. 2012; Chen et al. 2014; Pavlic-Zupanc et al. 2017; Burgess et al. 2019; Moral et al. 2019). It is, therefore, not surprising that they were isolated from Macadamia in both countries.

The largest group of isolates belonged to *Lasiodiplodia.* Species in the genus are found on a wide range of hosts including nut and fruits trees globally (Coutinho et al. 2017; Moral et al. 2019). *Lasiodiplodia pseudotheobromae* accounted for the highest percentage of isolates obtained in this study. This species was found in all the growing regions sampled and was also the dominant species isolated from Macadamia in Australia (Jeff-Ego and Akinsanmi 2019; Mohankumar et al. 2022). *Lasiodiplodia gilanensis* is reported on Macadamia for the first time but was present in low numbers. This species was first described from Iran from twigs of an unknown woody plant (Abdollahzadeh et al. 2010). However, it has since been reported in China

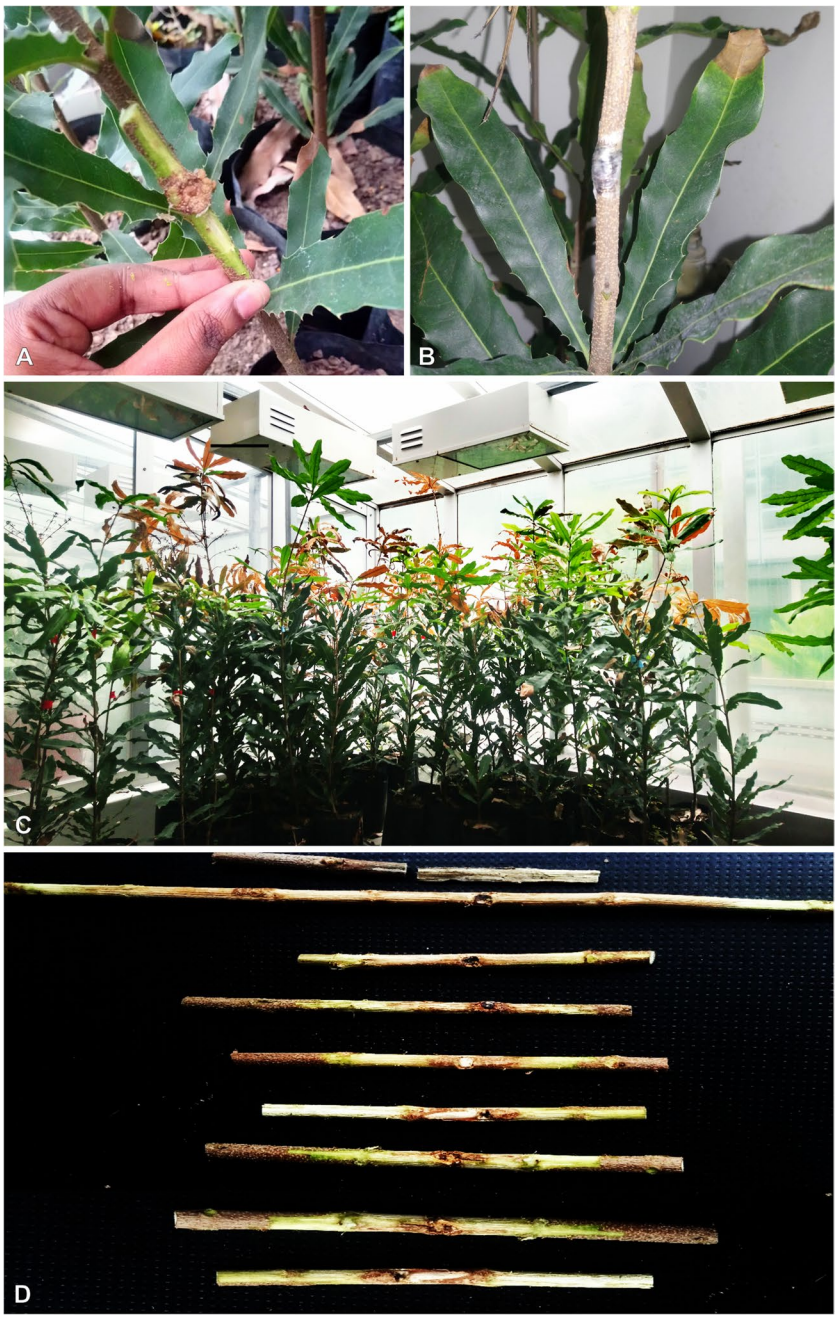

Fig. 6 Macadamia seedlings pathogenicity trials. (A) Seedlings nine weeks post inoculation forming a callus around the point of inoculation; (B) Dieback symptoms observed on leaves just below the point of inoculation. (C) Seedlings twelve weeks post inoculation showing typical branch dieback symptoms. (D) The difference in lesion length between the eight species used plus the control

(Li et al. 2018), Mexico (Rangel-Montoya et al. 2021) and the United States (Úrbez-Torres et al. 2017). In South Africa, *L. gilanensis* was first detected in KwaZulu-Natal on native *Syzygium cordatum* (Vivas et al. 2021). It is, therefore, possible that the species has moved between native *S. cordatum* and non-native Macadamia since they are often planted in close proximity to natural environments.

Hybridisation has been reported in a variety of important fungal pathogens. The present work reports three hybrids in *Lasiodiplodia*, which is currently the only genus in the *Botryosphaeriaceae* where hybridization has been described (Sakalidis et al. 2011; Cruywagen et al. 2017; Rodríguez-Gálvez et al. 2017). It is possible that some hybrids in other genera have been overlooked due to the reliance on ITS and *tef-1α* sequence data, as the identification of hybrids requires consideration of incongruence between multiple loci (Cruywagen et al. 2017). Hybridization events leads to the generation of unique genetic diversity that could result in speciation events, host range expansions or other ecological changes (Brasier and Kirk 2001). The presence and frequency of such hybrids should therefore be studied in more detail in future.

Neofusicoccum was the second most dominant genus present in this study. Out of the three species found in this

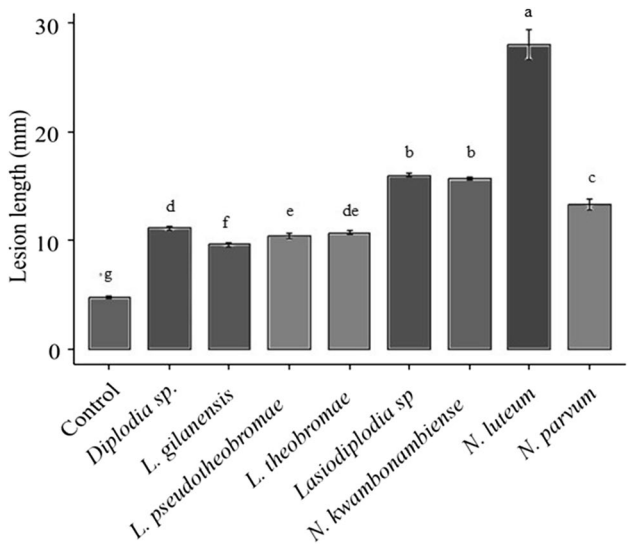

Fig. 7 Necrotic lesion length (mm) caused by isolates of *Botryosphaeriaceae* on one-year-old Beaumont plants. Bars with same letter are not significantly different from each other

study, *N. parvum* was the most common across the different regions in this study and in Australia (Jeff-Ego and Akinsanmi 2019; Mohankumar et al. 2022). In contrast, *N. luteum* was the least frequently isolated in Australia (Jeff-Ego and Akinsanmi 2019; Mohankumar et al. 2022) and in the current study. The second most prevalent species in this genus was *N. kwambonambiense,* which has been reported on various hosts in countries such as Brazil, South Africa, Uruguay and Australia (Pérez et al. 2008; Pavlic et al. 2009; Lopes et al. 2016; Liddle et al. 2019). However, despite its occurrence on other hosts in Australia, *N. kwambonambiense* has not previously been isolated from Macadamia.

This study is the first report of the genus *Diplodia* on Macadamia. Currently, the genus comprises of 25 species based on morphology and DNA sequence data of the ITS, *tef-1α*, and *tub2* gene regions (Dissanayake et al. 2016; Slippers et al. 2017; Yang et al. 2017; Zhang et al. 2021). The two isolates (CMW46906, CMW48899) obtained in the current study, however, could not be assigned to a specific species due to the lack of bootstrap support to confidently separate *D. insularis, D. alatafructa,* and *D. pseudoseriata* from one another. Our results are therefore in agreement to Zhang et al. (2021) and Phillips et al. (2012) which suggested that *D. alatafructa,* and *D. insularis* should be reduced to synonymy with *D. pseudoseriata*.

The comparison between asymptomatic and symptomatic trees revealed that six of the eight species found in this study occurred on both tissues. In addition, species unique to either tissue was also detected in low numbers and was unique to a specific area and/or farm. The results suggest a higher diversity of species present on symptomatic tissues than in asymptomatic tissues, in agreement to what was found in a survey from Macadamia branches in Australia (Mohankumar et al. 2022). Laurent et al. (2020), however, used a metabarcoding strategy to study the health status of *Quercus, Vitis* and *Pinus* and found no significant difference in species diversity (Laurent et al. 2020). This study is in agreement with the results of Cruywagen et al. (2017) where there was no distinction in the species diversity between asymptomatic and symptomatic baobab trees. This is however contrary to the study by Jami et al. (2013) where they found a greater species diversity in asymptomatic branches of *Acacia karoo* in South Africa. It is clear from these studies that a number of species can be involved in disease symptoms on a particular host, but that it does not represent the full diversity of the *Botryosphaeriaceae* on the host.

All eight *Botryosphaeriaceae* species were able to cause lesions on Macadamia seedlings. Therefore, the ability of these species to cause disease was irrespective of whether the species was isolated from asymptomatic or symptomatic tissue. In addition, the results also suggests that the initiation of the pathogenic potential of the species occurred subsequent to the reduction in irrigation. The results presented here therefore aligns with the concept that *Botryosphaeriaceae* species are latent pathogens that can live in plants without causing any symptoms until some external factor, such as water stress, results in the alteration in the plant-pathogen interaction and induces a switch to a pathogenic state (Slippers & Wingfield 2007).

The *Botryosphaeriaceae* species differed in aggressiveness. *Neofusicoccum luteum* was the most aggressive species in our study and was also described as one of the most aggressive species on Macadamia in Australia (Jeff-Ego and Akinsanmi 2019; Mohankumar et al. 2022). The novel *Lasiodoplodia* sp. was the second most aggressive and should be monitored in future. *Neofusicoccum kwambonambiense* was the third most aggressive species and was also described as one of the most aggressive species on Macadamia leaves in Australia (Liddle et al. 2019). It is however also known that there is variation in species aggressiveness of the *Botryosphaeriaceae* and that isolates of the same species may differ in their aggressiveness (Moral et al. 2019). Future research should therefore include multiple isolates per species.

In this study, the aggressiveness of *Botryosphaeriaceae* species on Macadamia was not necessarily correlated with its isolation frequency. For example, *L. pseudotheobromae* was the most dominant species in both Australia and South Africa, however it was not the most aggressive species on Macadamia in both countries (Jeff-Ego and Akinsanmi 2019; Mohankumar et al. 2022). Similarly, this species was also frequently isolated in a study by Coutinho et al. (2017) from branches of hog plum, tamarind and cashew. However, its aggressiveness was lower compared to other species

when inoculated into mango fruit (Coutinho et al. 2017). To the contrary, *N. luteum* was found in low numbers but is very aggressive. The relative contribution of the different species could thus be influenced by both their frequency, as well as their aggressiveness, and both factors should be considered when considering management options.

In summary, this study expands the knowledge of the occurrence of *Botryosphaeriaceae* on commercially planted Macadamia trees in South Africa. The study emphasizes the extensive diversity of the *Botryosphaeriaceae* community that can exist on both asymptomatic and symptomatic Macadamia trees. Furthermore, all species showed a potential to cause branch dieback symptoms, with *Neofusicoccum* species identified as the most aggressive in this study, while *Lasiodiplodia* species were the most common. The rapid expansion of Macadamia plantations and the association of *Botryosphaeriaceae* with extreme weather conditions, raise concerns about the threat of *Botryosphaeriaceae*-related diseases in South Africa in the future (Desprez-Loustau et al. 2006; Slippers and Wingfield 2007). Future work should therefore focus on understanding the effect of stress on the disease expression of *Botryosphaeriaceae* species on different Macadamia cultivars. This should also include pathogenicity assays that mimic the natural penetration and infection process.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s13313-024-00992-6.

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Data availability All sequence data generated for this study have been submitted to GenBank (accession numbers are listed in Online Resource Table 1) and any additional data are available on request.

Declarations

Conflict of interest The authors have no conflict of interest to declare that are relevant to this article. 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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