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Mycobiome associated with macadamia plants at different growth stages

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Macadamia is a tree producing high-value nuts and is indigenous to subtropical Australia. Macadamia is commercially cultivated across the globe and the macadamia industry is experiencing increased crop losses caused by fungal pathogens. Limited information is available regarding the association of fungal microbes with macadamia nursery plants. Therefore, this study aims to characterise fungal microbes associated with macadamia plants across five growth stages in leaf, stem and root during four seasons. The fungal community in macadamia nursery plants was analysed using a culture-based approach. Fungi were isolated from germinated seedlings, 3-month-old seedlings, non-grafted plants, grafted trees and 2-year-old trees in orchard. A total of 63 fungal species were identified using DNA sequencing, mainly in the phylum Ascomycota, from the different plant organs. The fungal community structure was significantly influenced by the growth stages ($P = 0.001$) and plant organs ($P < 0.001$). There was no significant seasonal ($P = 0.021$) effect in the number of fungal genera isolated from the plants. Grafted plants and young orchards had the richest fungal composition and diversity of the 63 fungal species. *Alternaria*, *Arcopilus*, *Epicoccum*, *Nigrospora*, and *Preussia* were found in the leaf, *Nectria* in the stem, and *Mortierella* and *Penicillium* in the root organ only. *Diaporthe*, *Neopestalotiopsis*, *Pestalotiopsis*, *Fusarium*, and *Phyllosticta* were detected in all three organs. This study revealed that fungal community richness and diversity in macadamia plants depend on the growth stage and organ examined.

KEYWORDS

Ascomycota, fungal diversity, fungal richness, nursery, tree nut

1 Introduction

Macadamias (*Macadamia integrifolia* and *M. tetraphylla*, along with their hybrids) are tropical evergreen trees in the plant family Proteaceae and originated in the subtropical rainforest of eastern Australia (Hardner et al., 2019). Macadamia is cultivated for its high-value edible nut and is the only Australian native food crop that has been cultivated on a commercial scale worldwide (Nock et al., 2019). Macadamia nut global demand is increasing, and therefore, the macadamia industry is rapidly expanding worldwide, with

a total estimated production of over 78,000 metric tons (kernel basis) in 2020 (INC, 2023). Macadamia is mostly cultivated in Australia, South Africa, USA, China, Kenya, Guatemala, Vietnam, Malawi, Zimbabwe, Brazil, Mexico, Columbia, Mozambique, Paraguay, Rwanda, and New Zealand (INC, 2023). In Australia, major commercial macadamia orchards are found in the Northern Rivers and Nambucca regions of New South Wales (NSW) and Queensland (QLD) in Mackay, Emerald, Bundaberg, Maryborough, and Glasshouse Mountains. The Australian macadamia industry is rapidly expanding, particularly in the Bundaberg region. Macadamia trees occupy over 32,400 hectares under cultivation in Australia, with an annual production of 15,840 metric tons of kernels (AMS, 2024; INC, 2023).

A preliminary study on the macadamia microbial profile shows that nursery plants are inhabited by several fungal genera (Sosso et al., 2021). The structure and function of fungal communities may depend on numerous variables, including plant growth stage, season, planting density, availability of nutrients, environmental conditions, and interactions with the surrounding ecological microorganisms (Liu and Howell, 2021; Gupta et al., 2022; Wei et al., 2023; Habtewold et al., 2021). The variability of fungal communities between seasons and plants under field conditions is usually very high compared to controlled conditions (Hannula et al., 2012). The impact of fungal communities on plants may be (1) direct (expression of disease symptoms or plant death) or (2) indirect (unintended changes in the metabolism of plants, thereby affecting overall growth and production) (Hannula et al., 2012; Birch et al., 2007). The importance of the effects exerted by these factors is still largely unknown in macadamia plants.

Previous studies in other crops have focused on one growth stage and one season without investigating variability over seasons in the crops (Hannula et al., 2012; Negi et al., 2024; Habtewold et al., 2021). In macadamia, a preliminary study showed possible variation in fungal populations in macadamia seedlings and grafted plants (Sosso et al., 2021). However, there is a dearth of information on the fungal diversity at different growth stages (0–3 months old seedlings, 3–6 months old established seedlings, 6–12 months old non-grafted plants, 18–24 months old grafted plants, and 0–2 years old established orchard), seasons (Spring, Summer, Autumn, and Winter) and plant organ (Leaf, Stem, and Root). The fungal communities within plant systems change in response to plant development (Copeland et al., 2015) and have a significant impact on plant health and production (Zahid et al., 2021). Identifying the normal variation in fungal communities during plant growth stages and seasons is crucial to evaluating crop health, yield potential, and potential shifts from endophytic behavior to pathogenic behavior of fungal compositions.

It is not well understood how these non-pathogenic endophytes turn to pathogenic mode in plants (Collinge et al., 2022). The switch of endophytes into pathogenic microbes might be caused by environmental or plant stress (Eaton et al., 2011; Collinge et al., 2022). Extensive research has been conducted on endophytes in agricultural grass hosts as well as a few forest trees like maple, red alder, oak, and various conifers (Wolfe et al., 2018; Szink et al., 2016; Liao et al., 2025; Wolfe et al., 2022; Nicoletti et al., 2021; Fernandez-

Conradi et al., 2019; Coelho et al., 2023). However, no research has been conducted on macadamia.

Given that the establishment and prevalence of plant–endophyte interactions are still unknown in macadamia, we sought to examine the fungal composition in batches of plants from germinated seedlings in the nursery to field establishment to answer the following research questions: (1) which fungi persist throughout the plant growth stages in different seasons and organs?, (2) which fungi are found in macadamia nursery plants?, (3) do fungal communities differ in macadamia nursery plants at different growth stages?, (4) if these species are vertically or horizontally transmitted in macadamia, and (5) what is the impact of resident endophytes on field establishment of macadamia plants. It is hypothesized that various fungal microbes infect and inhabit macadamia plants at various growth stages and seasons in nurseries in Australia. Knowledge of the species present and insight how this fungal community can be manipulated may be useful to improve productivity.

2 Materials and methods

2.1 Plant samples

To determine the fungal diversity in leaf, stem, and root tissues at different growth stages and seasons, macadamia plants were sampled from selected batches (Table 1) in Bundaberg, Queensland (24° 51' 58" S, 152° 20' 52" E; altitude 17.1 m). Sequential sampling was conducted to represent four growth stages including germinated seedlings (<3 months old), established seedlings (3–6 months old), non-grafted plants (6–12 months old) and grafted plants (18–24 months old). In addition, samples were obtained from two batches, 3 months after field establishment in an orchard (Table 1). A total of 170 plants were sampled. Each plant sample was carefully washed under running tap water, to remove debris from the surface. Leaf, stem, and root tissues were obtained from each sample of germinated seedlings (n = 10), established seedlings (n = 15), non-grafted plants (n = 20), grafted plants (n = 95), and young grafted trees in the orchard (n = 30).

2.2 Fungal isolation

Approximately 5–10 mm disc of each tissue segment was used for microbial isolation. A total of five tissues pieces per plate in three replicates were used for each sample. Surface sterilisation of the tissues was conducted by soaking in 70% ethanol (w/v) for 2 min, followed by 3% sodium hypochlorite solution (NaOCl) containing 0.1% Tween 20 for 2 min and in 50% ethanol (w/v) for 2 min (Araújo et al., 2001). Tissues were then rinsed twice in sterile water (Millipore Q-POD® Milli-Q, Biopak®) for 3 min and dried on sterile blotting paper before planting on 2% MEA (Malt extract agar, pH 5.5) (Merck KGaA, Darmstadt, Germany) in 90 mm plastic Petri plates. The plates were incubated at 25 °C with a 12-h photoperiod for 3–5 days. All fungal colonies were subcultured on

TABLE 1 Macadamia plants sampled at various growth stages and propagation batches.

Growth stage	Sampling season*					Total number of samples
	Spring 2021	Summer 2021-2022	Autumn 2022	Winter 2022	Spring 2022	
Germinated seedlings (<3 months-old)	Batch 1					10
Established seedlings (3–6 months-old)	Batch 2	Batch 1				15
Non-grafted plants (6–12 months-old)	Batch 3	Batch 2	Batch 1			20
Grafted plants (18–24 months-old)		Batch 3	Batch 2	Batch 1		20
	Batch 4	Batch 4	Batch 3	Batch 2	Batch 1	30
	Batch 5	Batch 5	Batch 4	Batch 3	Batch 2	25
	Batch 6	Batch 6		Batch 4	Batch 3	20
Young grafted trees (0–2 years-old orchard)			Batch 5	Batch 5	Batch 5	15
			Batch 6	Batch 6	Batch 6	15
Total	35	35	35	35	30	170

*Spring (September–November), Summer (December–February), Autumn (March–May), Winter (June–August). The total number of plants sampled per season is indicated in bold. The sampling design consisted of 10 plants per season from Batch 1 and 5 plants per season from all other batches.

½-strength PDA (Potato dextrose agar, pH 7.0) (Difco™ Becton, Dickinson and Company, Sparks, USA) plates amended with streptomycin (50 µg/ml). Monoconidial and single hyphal tip cultures were derived from selected isolates as described by Akinsanmi et al. (2004) and stored at -80°C in sterile 15% glycerol solution (Akinsanmi et al., 2004).

2.3 Molecular characterisation of fungal isolates

For identification of the fungal isolates, the DNA was extracted from mycelium (≈40 mg) using robotic platform by following the procedure of BioSprint 96 DNA Plant Kit (Qiagen Pty Ltd), after the mycelium tissues was homogenised in 300 µl RLT lysis buffer with a 3 mm sterile tungsten carbide bead for 2 min at 30 Hz using TissueLyser (Qiagen Pty Ltd). Biodrop (Duo Spectrophotometer) was used for DNA quantification and the final concentration was maintained at 10 ng/µl. Polymerase Chain Reaction (PCR) was performed to amplify a portion of the 18S, 5.8S, and 28S nrRNA of the internal transcribed spacer (ITS) region using primers ITS4 and ITS5 as fungal barcoding gene (White et al., 1990). The fungal microbes identity were confirmed by multi-gene analyses of its sequences with the β-tubulin (BT) sequence of gene region using T1 and BT2b primers (Glass and Donaldson, 1995) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with primers GDF and GDR (Templeton et al., 1992); partial actin (ACT) using ACT-512F and ACT-783R (Carbone and Kohn, 1999); and translation elongation factor 1-alpha (TEF) gene with EF1-688F and EF1-1251R primers (Alves et al., 2008). PCR reaction mixes were composed of 10 µl 5X Mangotag reaction buffer, 4 µl MgCl₂, 1 µl dNTPs, 1 µl of each forward and reverse primer, 2 µl BSA (10 µg/µl), 1 µl 100% DMSO,

0.4 µl DNAtaq Polymerase, 26.6 µl of DNase free water and 3 µl template DNA, totaling 50 µl. The PCR was performed in a Thermal Cycler (Kyrattech, Fisher Biotech, Australia). Initial denaturation was performed at 95°C for 5 min, denaturation at 95°C for 30 s, annealing for ITS and BT at 49°C for 30 s, GAPDH and ACT at 61.5°C for 30 s, and for TEF at 52°C for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 5 min. PCR amplicons were stained with 3X GelRed and separated on 1.0% agarose gels (Bioline, Australia) in 0.5% tris-ethylenediaminetetraacetic acid (TE) buffer and electrophoresis at 100V for 45 min. Successful amplification was determined under ultraviolet (UV) transillumination using Molecular Imager® and GelDoc™ (Bio-Rad Laboratories Inc., Segrate, and Milan, Italy). A 1 kb HyperLadder (Bioline) was used to deduce amplicon size. Both a negative control with no template DNA, and a positive control were included. Amplified products were purified using QIAquick PCR Purification Kit (Qiagen) according to manufacturer's instructions and sequenced in both direction at MacroGen Inc. (South Korea). DNA sequences were manually trimmed, checked for quality in Geneious software version 10.2.4 (Biomatters, Ltd., Auckland, New Zealand) and compared with sequences in GenBank nucleotide database using Blast Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov/>) in National Centre for Biotechnology Information (NCBI). Sequences with pairwise similarities > 99% were used to determine the identity of the fungal isolates.

2.4 Impact and translation to field disease

To understand the impact of endophytes on plant diseases that lead to early tree mortality in young orchards, a disease survey was conducted for macadamia plant batch 5 (A203/H2) and batch 6

(HAES741/H2) transplanted into the orchard during the study. Both batches were transplanted into the same farmer's orchard and kept under similar management practices. The macadamia plants were surveyed for visual symptoms of stem diseases, including graft dieback, stem canker, gall canker and tree mortality during 2022–2023. In each orchard during each visit, 10 rows and 20 consecutive plants from each row were observed for stem diseases. The trees with symptoms were tagged and excluded from the follow-up visit. A total of 800 trees, each of batch 5 and batch 6, were surveyed. The four border rows of each end of the orchard were excluded from the study to eliminate field border effects.

2.5 Biocontrol potential of macadamia endophytes

Macadamia endophytic isolates were evaluated *in vitro* to understand the potential antagonistic activity against known pathogens. Representative isolates of *Aspergillus japonicus*, *A. hirsutiae*, *A. ibericus*, *Phyllosticta paracapitalensis*, *P. capitalensis*, *Mortierella elongate*, *Trichoderma atroviride*, *T. koningii*, and *T. longibrachiatum* were evaluated for their antagonistic activity against known stem pathogens, *Diaporthe australiana*, *Neofusicoccum luteum*, *N. parvum*, *Lasiodiplodia theobromae*, *L. pseudotheobromae*, *L. iraniensis* and *Nectria pseudotrichia*. Briefly, mycelial discs of 5 mm diameter from the edge of actively growing colonies of each antagonist and pathogen were placed at opposite sites on a 90 mm diameter PDA plate (20 mm from the border). The pathogen alone on the plate was used as a control. The plates were incubated at 25°C with a 12-h photoperiod for 7 days. After 7 days, the radius of pathogen colonies facing the antagonists was measured and used to calculate the mycelial growth inhibition rate. The antagonistic levels were classified as low ($\leq 50\%$), medium (50% - 60%), high (60% - 75%) and very high ($>75\%$).

2.6 Statistical analysis

Fungal population data for plant organs, plant growth stages, seasons, and interactions among plant organs, growth stage, and seasons were analysed by general analysis of variance (ANOVA) procedure in GenStat 23rd edition (VSN International, Hemel Hempstead, UK). The isolation rate (%) of each fungal species was calculated using the following formula

$$\text{Isolation rate (\%)} = \frac{A}{S} \times 100$$

A is the number of isolates from the same species, and S is the total number of isolates.

Fungal population diversity measures, including richness, Shannon Weaver evenness index (J') and Shannon Weaver diversity index (H'), were calculated for each plant organs, growth stage, and seasons separately. The H' was calculated using the

following formula:

$$H' = -\sum_{i=1}^s P_i \times \ln(P_i)$$

Where P_i is the proportion of individuals of a particular taxon (i), s is the total number of isolates and $\ln$ is the natural logarithm.

The Shannon Weiner evenness (J') was calculated using following formula:

$$J' = \frac{H'}{\log(s)}$$

Where H' is the Shannon index and s is the total number of isolates.

The stem Disease Prevalence (DP) was based on the number of symptomatic plants relative to the cumulative number of plants sampled within batch 5 and batch 6 plants that were transplanted into orchards using the following formula:

$$DP = \frac{\text{Number of symptomatic plants}}{\text{Total number of plant sampled}} \times 100$$

To validate the relationship of fungal species within batch 5 and batch 6, macadamia plants were explored using the principal component analysis procedure in GenStat 23rd edition.

The mycelial growth inhibition (%) of pathogens was calculated using the following formula,

$$\text{Mycelial growth inhibition (\%)} = \frac{Pr - Ar}{Pr} \times 100$$

Where Pr is the growth radius of the pathogen in control, and Ar is the growth radius of the pathogen with antagonists.

3 Results

3.1 Fungal microbes in macadamia

In this study, 5874 fungal microbes were isolated from macadamia plants in Australia (Table 2). A total of 14 fungal families consisting of 20 genera and 63 species were identified from macadamia plant samples. The majority of isolates belonged to the *Diaporthe* (15.8%) followed by *Neopestalotiopsis* (13.3%), *Colletotrichum* (11.1%), *Alternaria* (6.8%), *Pestalotiopsis* (6.2%), *Cladosporium* (5.9%), *Phyllosticta* (5.0%), *Neofusicoccum* (4.6%), and *Lasiodiplodia* (4.1%) while a low frequency of isolation was observed for *Paraconiothyrium* (0.8%) (Table 2; Figure 1). At the species level, isolation frequencies ranged from 0.2% to 4.2%. *Colletotrichum siamense* and *C. gloeosporioides* were the most common (4.2% each), followed by *N. clavispora* (3.8%), whereas *Arcopilus navicularis* was the least frequent (0.2%) (Table 2; Figure 1).

Statistical analysis revealed that both growth stage and plant organ had a highly significant effect on fungal community structure ($P < 0.001$ for both). Although season also showed a significant effect ($P = 0.021$), its influence was substantially weaker than that of

TABLE 2 Fungal species obtained from macadamia nursery plants at different growth stages.

Fungal species	Growth stage					Total	Isolation rate (%)
	Germinated seedlings	Established seedlings	Non-grafted plants	Grafted plants	Young grafted trees		
<i>Alternaria alternata</i>		28	41	95	30	194	3.3
<i>Alternaria tenuissima</i>		24	35	100	46	205	3.5
<i>Arcopilus aureus</i>				32	18	50	0.9
<i>Arcopilus navicularis</i>				7	4	11	0.2
<i>Aspergillus hiratsukae</i>			9	41	24	74	1.3
<i>Aspergillus ibericus</i>	4	3	4	31	31	73	1.2
<i>Aspergillus japonicus</i>	3	6	10	29	22	70	1.2
<i>Colletotrichum alienum</i>		8	8	5	6	27	0.5
<i>Colletotrichum boninense</i>		4	4	14	13	35	0.6
<i>Colletotrichum fruticola</i>		1	2	16	5	24	0.4
<i>Colletotrichum gloeosporioides</i>		10	21	133	80	244	4.2
<i>Colletotrichum karsti</i>		1	4	5	8	18	0.3
<i>Colletotrichum perseae</i>		3	4	9	10	26	0.4
<i>Colletotrichum siamense</i>		29	24	81	112	246	4.2
<i>Colletotrichum theobromicola</i>		6	6	12	6	30	0.5
<i>Cladosporium cladosporioides</i>				67	122	189	3.2
<i>Cladosporium pseudocladosporioides</i>				12	8	20	0.3
<i>Cladosporium psychrotolerans</i>				53	85	138	2.3
<i>Diaporthe australiana</i>				86	54	140	2.4
<i>Diaporthe fraxini-angustifoliae</i>	22	25	28	132	76	283	4.8
<i>Diaporthe masirevicii</i>	8	7	7	76	49	147	2.5
<i>Diaporthe middletonii</i>	11	12	15	35	24	97	1.7
<i>Diaporthe musigena</i>	4	1	8	51	32	96	1.6
<i>Diaporthe sojae</i>				16	42	58	1.0
<i>Diaporthe arecae</i>	5	8	10	43	40	106	1.8
<i>Epicoccum dendrobii</i>	1	1	5	21	7	35	0.6
<i>Epicoccum nigrum</i>	5	8	30	62	23	128	2.2
<i>Fusarium incarnatum</i>			17	74	70	161	2.7
<i>Fusarium proliferatum</i>			25	102	68	195	3.3
<i>Lasiodiplodia iraniensis</i>				13	10	23	0.4
<i>Lasiodiplodia pseudotheobromae</i>		9	7	64	20	100	1.7
<i>Lasiodiplodia theobromae</i>		9	13	62	32	116	2.0

(Continued)

TABLE 2 Continued

Fungal species	Growth stage					Total	Isolation rate (%)
	Germinated seedlings	Established seedlings	Non-grafted plants	Grafted plants	Young grafted trees		
<i>Mortierella elongata</i>			13	73	35	121	2.1
<i>Neopestalotiopsis acrostichi</i>		5	9	9	21	44	0.7
<i>Neopestalotiopsis australis</i>			7	15	14	36	0.6
<i>Neopestalotiopsis clavispora</i>			52	106	66	224	3.8
<i>Neopestalotiopsis elaeidis</i>			4	12	14	30	0.5
<i>Neopestalotiopsis foedans</i>			6	17	17	40	0.7
<i>Neopestalotiopsis macadamiae</i>			23	37	49	109	1.9
<i>Neopestalotiopsis maddoxii</i>			30	74	67	171	2.9
<i>Neopestalotiopsis nebuloides</i>			7	17	11	35	0.6
<i>Neopestalotiopsis pernambucana</i>			9	14	16	39	0.7
<i>Neopestalotiopsis rosae</i>		5	5	8	7	25	0.4
<i>Neopestalotiopsis sonneratae</i>			6	15	9	30	0.5
<i>Nectria pseudotrichia</i>				113	18	131	2.2
<i>Neofusicoccum australe</i>				7	21	28	0.5
<i>Neofusicoccum luteum</i>		13	15	56	23	107	1.8
<i>Neofusicoccum mangroviorum</i>		1	5	17	14	37	0.6
<i>Neofusicoccum parvum</i>		5	16	52	25	98	1.7
<i>Nigrospora zimmermanii</i>	6	15	21	16	34	92	1.6
<i>Pestalotiopsis macadamiae</i>			9	11	30	50	0.9
<i>Pestalotiopsis paeoniicola</i>			13	43	58	114	1.9
<i>Pestalotiopsis telopeae</i>			15	16	49	80	1.4
<i>Pestalotiopsis versicolor</i>			17	43	60	120	2.0
<i>Paraconiothyrium brasiliense</i>				27	22	49	0.8
<i>Penicillium limosum</i>			2	15	22	39	0.7
<i>Penicillium pinophilum</i>	4	6	4	28	28	70	1.2
<i>Phyllosticta capitalensis</i>	2	6	18	69	59	154	2.6
<i>Phyllosticta paracapitalensis</i>	2	5	12	65	56	140	2.4
<i>Preussia africana</i>				45	29	74	1.3
<i>Trichoderma atroviride</i>		6	8	29	12	55	0.9

(Continued)

TABLE 2 Continued

Fungal species	Growth stage					Total	Isolation rate (%)
	Germinated seedlings	Established seedlings	Non-grafted plants	Grafted plants	Young grafted trees		
<i>Trichoderma longibrachiatum</i>		1	2	70	6	79	1.3
<i>Trichoderma koningii</i>		5	6	49	34	94	1.6
Total	80	276	668	2747	2103	5874	100

Bold values represent subtotals of isolates for each plant growth stage and the total across all stages sampled in macadamia nurseries.

growth stage and organ. The interaction between growth stage and plant organs ($P < 0.001$), growth stage and season ($P = 0.009$), and plant organs and season ($P < 0.001$) was significantly different, while the interaction was not significantly different among growth stage, plant organs and season ($P = 0.028$). The major influencing parameters between growth stage and season, plant organs and season, growth stage, plant organs and season interactions were growth stages and plant organs. The variations in fungal diversity between growth stages and plant organs were much higher compared to seasons.

3.2 Diversity of fungal communities at various growth stages in macadamia

The fungal community does change among the different growth stages. Germinated seedlings have the lowest diversity index ($H' = 2.36$), followed by established seedlings ($H' = 3.19$), non-grafted plants ($H' = 3.68$), grafted plants ($H' = 3.89$), and young grafted trees ($H' = 3.89$). The highest evenness index was in young grafted trees ($J' = 0.94$), grafted plants ($J' = 0.93$), and non-grated plants ($J' = 0.93$), followed by established seedlings ($J' = 0.91$), and germinated seedlings ($J' = 0.89$). Young grafted trees and grafted plants have the richest species composition (63 species), followed by non-grafted plants (52 species), established seedlings (34 species), and germinated seedlings (14 species).

Six fungal genera, *Diaporthe*, *Phyllosticta*, *Aspergillus*, *Epicoccum*, *Penicillium*, and *Nigrospora* were detected in all five growth stages (Table 2). Six fungal genera, *Neopestalotiopsis*, *Colletotrichum*, *Neofusicoccum*, *Alternaria*, *Lasiodiplodia*, and *Trichoderma* were detected in four growth stages (Table 2). Three fungal genera, *Pestalotiopsis*, *Fusarium*, and *Mortierella* were detected in three growth stages, while *Cladosporium*, *Nectria*, *Preussia*, *Arcopilus*, and *Paraconiothyrium* were detected only in grafted plants and young grafted trees (Table 2; Figure 1). *Diaporthe* was the major genus identified in young grafted trees and grafted plants followed by *Neopestalotiopsis* and *Colletotrichum* (Table 2). In non-grafted plants and established seedlings, the major microbial genera were *Neopestalotiopsis*, *Colletotrichum*, *Alternaria*, and *Diaporthe*. In germinated seedlings, *Diaporthe* was the most prominent fungal genera (Table 2; Figure 1).

3.3 Species distribution at various growth stages in macadamia

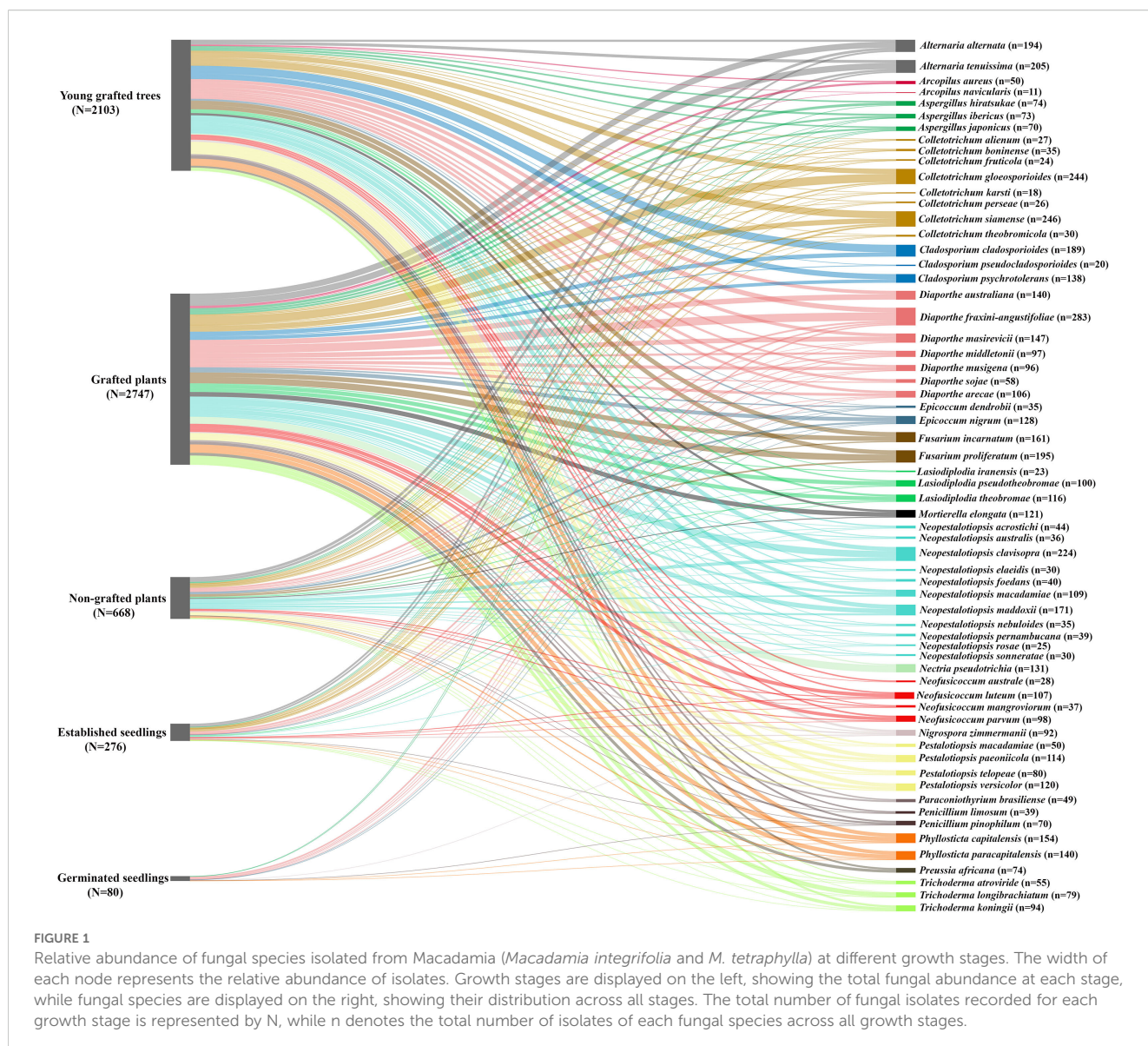
Thirteen fungal species were detected in all five growth stages, including *Diaporthe fraxini-angustifoliae*, *D. middletonii*, *D. masirevicii*, *D. sojae*, *D. musigena*, *Phyllosticta capitalensis*, *P. paracapitalensis*, *Aspergillus ibericus*, *A. japonicus*, *Epicoccum nigrum*, *E. dendrobii*, *Penicillium pinophilum*, and *Nigrospora zimmermanii* (Table 2). *Diaporthe australiana*, *D. sojae* and *D. litchicola* were detected only in grafted plants and young grafted trees, whereas *A. hiratsukae*, *P. limosum* were detected in non-grafted, grafted and young grafted trees (Table 2; Figure 1).

Neopestalotiopsis acrostichi, *N. rosae*, *Colletotrichum. alienum*, *C. boninense*, *C. fruticola*, *C. gloeosporioides*, *C. karsti*, *C. perseae*, *C. siamense*, and *C. theobromicola*, *Neofusicoccum luteum*, *N. mangroviorum*, *N. parvum*, *Alternaria alternata*, *A. tenuissima*, *Lasiodiplodia pseudotheobromae*, *L. theobromae*, *Trichoderma koningii*, *T. atroviride*, and *T. longibrachiatum* were detected in four growth stages, established seedlings, non-grafted plants, grafted plants, young grafted trees. *Neopestalotiopsis australis*, *N. clavispora*, *N. elaeidis*, *N. foedans*, *N. macadamiae*, *N. maddoxii*, *N. nebuloides*, *N. pernambucana*, and *N. sonneratae* were detected in non-grafted, grafted plants and young grafted trees in the field. *Neofusicoccum australe* and *L. iraniensis* were detected in grafted plants and young grafted trees (Table 2; Figure 1).

Pestalotiopsis macadamiae, *P. paeoniicola*, *P. telopeae*, *P. versicolor*, *Fusarium incarnatum*, *F. proliferatum* and *Mortierella elongate* were detected in three growth stages: non-grafted plants, grafted plants and young grafted trees (Table 2). *Cladosporium cladosporioides*, *C. psychrotolerans*, *C. pseudocladosporioides*, *Nectria pseudotrichia*, *Preussia africana*, *Arcopilus aureus*, *A. navicularis*, and *Paraconiothyrium brasiliense* were detected only in grafted plants and young grafted trees (Table 2).

3.4 Fungal diversity in macadamia leaf, stem, and root of plants

Stem tissues had the highest diversity index ($H' = 3.63$) followed by leaf ($H' = 3.44$) and root ($H' = 3.31$). While the evenness index was highest in stem ($J' = 0.92$) and root ($J' = 0.92$), followed by leaf



($J' = 0.88$). A total of 52 species were obtained from the stem, followed by 51 species from the leaf and 36 species from the root.

Five fungal genera *Diaporthe*, *Neopestalotiopsis*, *Pestalotiopsis*, *Fusarium*, and *Phyllosticta* were detected in all three organs. At the species level, *D. australiana*, *D. sojae*, and *F. incarnatum* were detected in the stem and root. *Fusarium proliferatum* was detected in the leaf and stem (Figure 1), and the remaining species were detected in all three organs (Figure 2).

Four fungal genera, *Colletotrichum*, *Cladosporium*, *Neofusicoccum*, *Lasiodiplodia*, and all of their associated species were detected in leaf and stem organs. Accordingly, three fungal genera, *Trichoderma*, *Aspergillus*, and *Paraconiothyrium*, were detected in the stem and root.

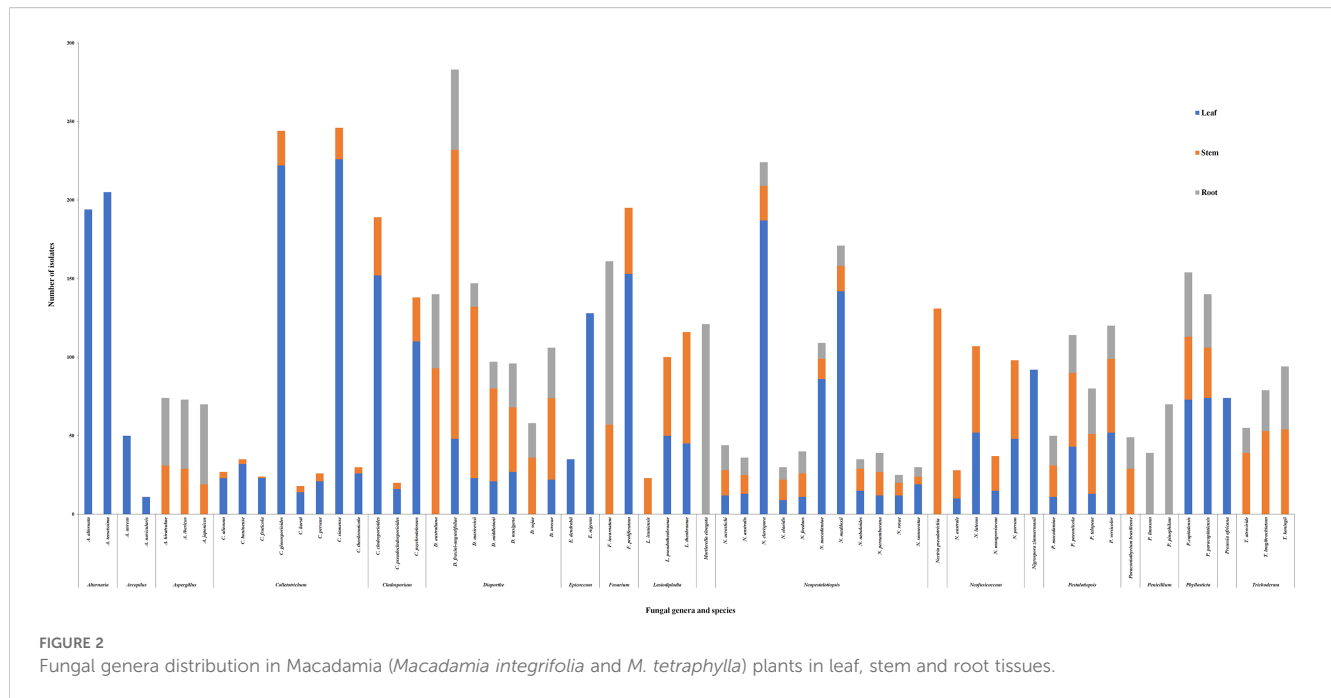
Alternaria, *Arcopilus*, *Epicoccum*, *Nigrospora*, and *Preussia* were detected in leaves only. *Nectria pseudotrichia* were detected in the stem, and *Mortierella* and *Penicillium* were detected only in the roots organ only (Figure 2).

3.5 Seasonal variation among fungal communities in nursery plants

Overall, the diversity and evenness indices among the four seasons (Spring, Summer, Autumn, and Winter) are strikingly similar. All 63 species were detected in autumn, winter, and spring, while 61 species were detected in summer.

3.6 Field disease impact

There was a significant difference in stem disease prevalence and tree mortality between batch 5 (A203/H2) and batch 6 (HAES741/H2). Batch 5 shows 4% of plants with graft dieback, 1% stem canker, 2% gall canker, and 7.5% tree mortality. In contrast, 9.5% of plants with graft dieback, 2.5% stem canker, 2.5% gall canker, and 13.5% tree mortality were in batch 6.



When comparing the fungal microbial data of both batches post-grafting stages, the isolation for *Trichoderma* spp. is significantly lower for batch 6 ($n = 10$) than batch 5 ($n = 93$), and the number of potential pathogenic *Neofusicoccum parvum* ($n = 33$), *N. luteum* ($n = 34$), *Lasiodiplodia theobromae* ($n = 48$), *L. pseudotheobromae* ($n = 32$), and *L. iraniensis* ($n = 18$) were significantly higher in batch 5 as compared to batch 6, *N. parvum* ($n = 9$), *N. luteum* ($n = 13$), *L. theobromae* ($n = 6$), and *L. pseudotheobromae* ($n = 2$).

3.7 Relationship among fungi

To explore the interactions among plant pathogenic and beneficial fungal species, a principal component analysis (PCA) was conducted using isolates from the stem and root organs of batch 5 and 6. The first two principal components explained 71.5% of the variation. The PC1 separated the beneficial fungi from the pathogenic fungi (Figure 3). The PCA analysis revealed the beneficial fungi were separated from the pathogen fungal species, based on their aggressiveness or frequency of colonisation in the stem (Figure 3). The pathogenic species of *Neofusicoccum parvum*, *N. luteum*, *Lasiodiplodia theobromae*, *L. pseudotheobromae*, and *L. iraniensis* were clustered together and were segregated from *Diaporthe australiana*, *Nectria pseudotrachia* (Figure 3). The occurrence of *Diaporthe australiana* and *Nectria pseudotrachia* were unrelated to *Neofusicoccum*, *Lasiodiplodia*, and *Trichoderma* species (Figure 3).

Trichoderma atroviride, *T. koningii*, *T. longibrachiatum*, *Aspergillus ibericus*, *A. japonicus*, and *A. hiratsukae* co-occurred. These colonisers had a negative relationship with *Diaporthe australiana*, *Neofusicoccum. parvum*, *N. luteum*, *Lasiodiplodia theobromae*, *L. pseudotheobromae* and *L. iraniensis* graft dieback

and branch dieback pathogens (Figure 3). *Phyllosticta paracapitalensis*, *P. capitalensis* and *Mortierella elongate* seemed to co-occur and negatively related to *Neofusicoccum parvum*, *N. luteum*, *Lasiodiplodia theobromae*, *L. pseudotheobromae*, *L. iraniensis*, and *Nectria pseudotrachia* (Figure 3).

3.8 Biocontrol potential of macadamia plant endophytes

In vitro biological control of representative known macadamia pathogens *Diaporthe australiana*, *Neofusicoccum luteum*, *N. parvum*, *Lasiodiplodia theobromae*, *L. pseudotheobromae*, and *L. iraniensis* by potential biocontrol agents *Aspergillus japonicus*, *A. hiratsukae*, *A. ibericus*, *Phyllosticta paracapitalensis*, *P. capitalensis*, *Mortierella elongate*, *Trichoderma atroviride*, *T. koningii* and *T. longibrachiatum* by inhibition of mycelial growth of pathogens. All potential biocontrol agent isolates did not significantly reduce the mycelial growth of pathogens in dual culture tests in Petri dishes (Table 3).

Some good combinations of biocontrol agents were observed. A dual culture test in Petri dishes shows that *Trichoderma koningii*, *T. atroviride*, *Aspergillus ibericus* and *T. longibrachiatum* reduce the growth of *Diaporthe australiana* by 75.8%, 72.7%, 72.1% and 59.4%, respectively (Table 3).

4 Discussion

This study advanced understanding of the mycobiome in macadamia production systems, highlighting key shifts from seedling germination to young orchard trees. We identified a complex community of 63 fungal species across 20 genera,

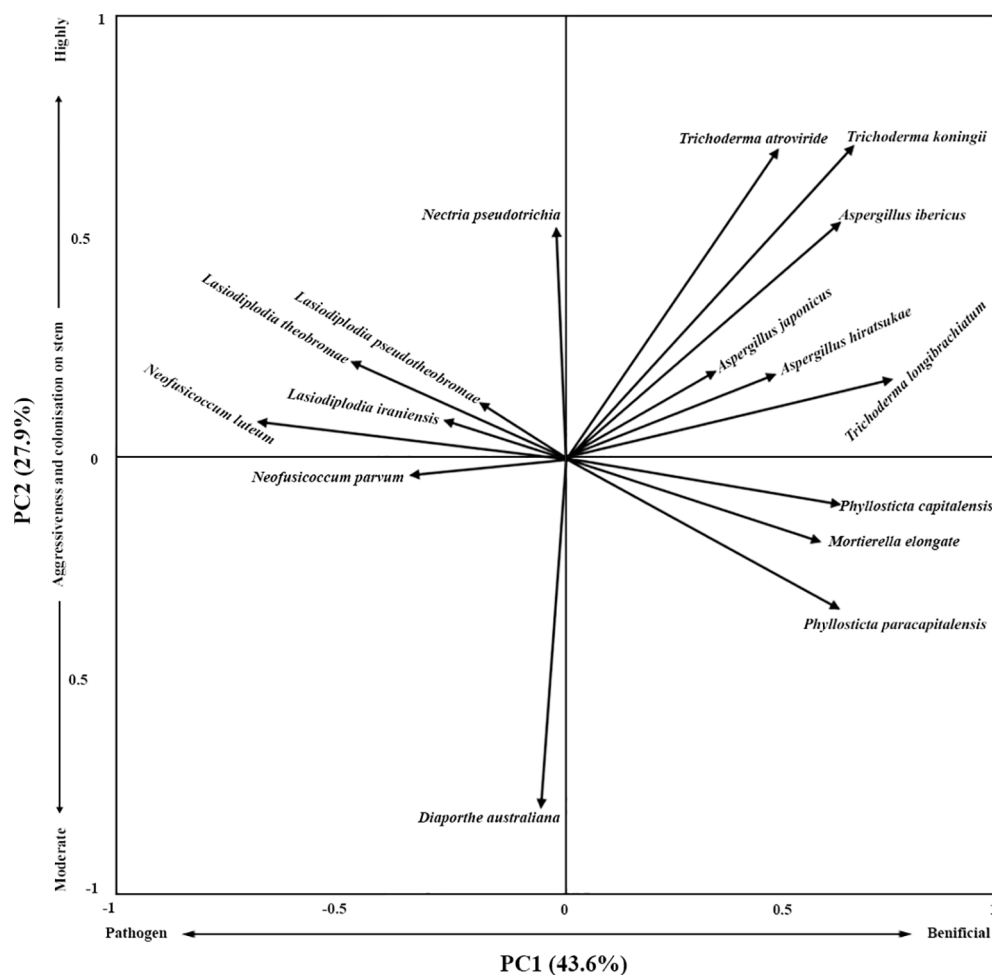


FIGURE 3

Ordination biplots from PCA analysis showing relationships between beneficial fungal (*Trichoderma atroviride*, *T. koningii*, *T. longibrachiatum*, *Aspergillus ibericus*, *A. japonicus*, *A. hirsutiae*, *Phyllosticta paracapitalensis*, *P. capitalensis*, and *Mortierella elongate*) and pathogenic species (*Neofusicoccum parvum*, *N. luteum*, *Lasiodiplodia theobromae*, *L. pseudotrachia*, *L. iraniensis*, *Diaporthe australiana*, and *Nectria pseudotrachia*).

revealing that this community is dynamic and shaped primarily by plant growth stage and organ type, rather than seasonal variation. Crucially, the *in vitro* antagonism assays, coupled with field disease data and interaction analyses, identified specific endophytic fungi with significant potential as native biocontrol agents against key macadamia pathogens.

Our findings clearly demonstrate that the macadamia mycobiome is not static but undergoes significant succession as the plant develops. The low diversity in germinated seedlings, dominated by a few genera like *Diaporthe*, suggests an initial colonisation by generalist fungi. The subsequent increase in diversity and evenness through established seedlings, non-grafted, and grafted plants reflects a process of ecological succession, where the developing plant recruits a more complex and likely more specialized microbial community (Hardoim et al., 2015). The convergence of high species richness in grafted plants and young trees indicates the establishment of a stable, mature mycobiome. The significant influence of plant organ, with stems harboring the

greatest diversity, highlights the concept of tissue specific micro-niches, where different anatomical sites select for distinct fungal communities based on factors like nutrient availability and chemical defenses (Zimmerman and Vitousek, 2012). The relative insignificance of season contrasts with studies on foliar pathogens in annual crops, suggesting that in perennial nursery systems, the host developmental stage is a stronger driver of microbial assembly than short-term environmental fluctuations.

The dual culture assays were an effective primary screen to identify endophytes with direct antagonistic capabilities. The strong inhibition of *Diaporthe australiana* by isolates of *Trichoderma koningii* (75.8%), *T. atroviride* (72.7%), and notably *Aspergillus ibericus* (72.1%) is a key finding. The efficacy of *Trichoderma* spp., is consistent with their well-documented role as mycoparasites (Harman et al., 2004). However, the high antagonistic activity of *Aspergillus ibericus*, a less commonly reported biocontrol agent, points to the value of screening native mycobiomes for novel functional traits. The specificity of these interactions, where other

TABLE 3 Percent mycelial growth rate (%) of endophytic fungi against known macadamia stem pathogens.

Endophytic fungi	<i>Diaporthe australiana</i>	<i>Neofusicoccum luteum</i>	<i>Neofusicoccum parvum</i>	<i>Lasiodiplodia theobromae</i>	<i>Lasiodiplodia pseudotrichia</i>	<i>Lasiodiplodia iraniensis</i>	<i>Nectria pseudotrichia</i>
<i>Aspergillus japonicus</i>	26.4	8.0	3.4	6.7	2.1	1.4	1.6
<i>Aspergillus hirsutiae</i>	28.5	10.7	1.7	2.7	2.8	2.9	3.3
<i>Aspergillus ibericus</i>	72.1	3.6	3.4	5.3	4.2	2.9	4.9
<i>Phyllosticta capitalensis</i>	44.2	1.8	3.4	8.0	6.9	7.1	9.8
<i>Phyllosticta paracapitalensis</i>	34.0	5.4	9.5	6.7	2.8	3.6	8.2
<i>Mortierella elongate</i>	28.5	1.8	1.7	6.7	4.2	2.9	9.8
<i>Trichoderma atroviride</i>	72.7	17.9	15.5	18.7	18.1	17.1	13.1
<i>Trichoderma koningi</i>	75.8	28.6	32.8	29.3	29.2	30.0	14.8
<i>Trichoderma longibrachiatum</i>	59.4	12.5	12.1	10.7	9.7	7.1	16.4

endophytes showed negligible activity, underscores that biocontrol potential is a strain-specific attribute rather than a generic feature of a genus.

The *in vitro* findings are strongly supported by the field disease data and the Principal Component Analysis (PCA). The stark contrast in disease outcomes between Batch 5 and Batch 6 provides compelling correlative evidence. Batch 6, which suffered higher mortality and disease prevalence, had a significantly lower abundance of *Trichoderma* spp., and a concomitantly higher abundance of pathogenic *Neofusicoccum* and *Lasiodiplodia* species. This inverse relationship suggests that a robust community of beneficial fungi, particularly *Trichoderma*, may suppress pathogen populations *in planta*. The PCA biplot (Figure 3) statistically validates this inverse relationship, clearly segregating beneficial fungi (*Trichoderma* spp., *Aspergillus* spp., and *Phyllosticta* spp.), from the aggressive pathogens (*Neofusicoccum* spp., *Lasiodiplodia* spp., *Diaporthe* sp. and *Nectria* sp.). The clustering of beneficial fungi together suggests potential synergism or shared environmental preferences, while their negative correlation with pathogens indicates competitive exclusion or antagonism. The separate clustering of *Diaporthe australiana* and *Nectria pseudotrichia* from the main pathogen group is intriguing, suggesting they may occupy a different ecological niche or have a different, perhaps less aggressive, pathogenic lifestyle. The negative relationship of *Phyllosticta* and *Mortierella* with pathogens further expands the list of potential beneficial taxa beyond *Trichoderma*.

The higher disease pressure in Batch 6 cannot be attributed to a single pathogen but rather to an enrichment of a pathobiome, a consortium of multiple pathogens occurring simultaneously (Lamichhane and Venturi, 2015). The data suggest that the absence of a strong beneficial mycobiome may facilitate the proliferation of this pathobiome. Therefore, managing for tree health may be more effectively achieved by fostering a protective mycobiome rather than targeting individual pathogens.

The isolation of potent antagonistic endophytes native to macadamia and already adapted to its internal tissues highlights their potential as ideal candidates for probiotic inoculants, as successful colonisation not only establishes a protected niche but also confers resistance to subsequent fungal invasions by enhancing the primary coloniser’s ability to persist and resist replacement (John and White, 2017).

In conclusion, this research delineates the succession of the mycobiome in macadamia nurseries and identifies key growth stages where microbial communities are most susceptible to change. We have identified specific native endophytes, most notably *Trichoderma koningii*, *T. atroviride*, and *Aspergillus ibericus*, that exhibit strong *in vitro* antagonism against major pathogens, a finding supported by inverse correlations. These isolates represent excellent candidates for developing novel biocontrol strategies to mitigate stem canker and other diseases. Future research should priorities *in planta* validation, mechanistic elucidation, and the development of synergistic endophyte consortia to achieve broad-spectrum and durable disease suppression. Advancing scalable delivery strategies will be critical to enable effective colonisation and persistence, thereby establishing

microbiome-informed approaches for sustainable disease management in the macadamia industry.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

JK: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Writing – original draft, Writing – review & editing. AD: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. OA: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – review & editing, Formal analysis, Validation.

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