Retention of post-harvest residues enhances soil fungal biodiversity in *Eucalyptus* plantations

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ABSTRACT

Plantation forests have the potential to meet global economic and ecological objectives. The sustainability of plantations is influenced by a variety of above- and below-ground factors. Among these factors are soil-associated microbes, as they play a vital role in soil biogeochemical processes. However, when compared to natural forests, the microbial biodiversity associated with plantation soils is often less biodiverse due to anthropogenic disturbances. The loss of microbial biodiversity in plantation soils can be partially mitigated through better post-harvest residue management regimes. To study this hypothesis in South African plantation soils, the effects of three post-harvest residue management regimes on fungal diversity were compared: retained, removed, or removed and fertilized. We collected 108 soil samples from these treatments at three study sites planted with a single *Eucalyptus* genotype. Consequently, DNA was extracted from all soil samples and fungal amplicon libraries were constructed using the Illumina MiSeq platform. The bioinformatic and statistical analyses of the data indicated that the post-harvest residue management regimes, the location of the study sites, and their interaction (soil treatment × site) influenced the community composition of soil fungi. The plots retaining post-harvest residues had a higher diversity of saprotrophs and symbiotrophs and fewer pathotrophs. The results showed that retaining post-harvest residue can improve the diversity of beneficial soil fungi in South African plantation soil. In the future, the South African and global forestry sectors should consider retaining post-harvest residues to ameliorate the tree and soil health of their plantations.

Keywords Soil microbiome, fungal diversity, plantation forestry, post-harvest residue management

1. Introduction

Monocultures of fast-growing trees are a major source of forest-based products including bioenergy, pulp, timber, and lignocellulose (Turnbull, 1999; Gomes *et al.*, 2021). With the growing demand for these products, the global plantation area is rapidly expanding. Plantations are also important carbon sinks because they offset the effect of greenhouse gases, which are a major contributor to climate change (Booth, 2013; Zhang *et al.*, 2017; Nunes *et al.*, 2019). Many plantation trees such as *Eucalyptus* are effective reforestation species and also act as refugia for floral and faunal diversity (Ramírez-Mejía and Sánchez, 2016; Santos *et al.*, 2020). Thus, the long-term sustainability of global plantations has the potential to meet both economic and ecological objectives.

Long-term sustainability of plantations can be achieved through improving tree health, which is considerably influenced by both above- and below-ground biotic and abiotic components. Two important below-ground components that influence plant health are soil nutrients and microbes (Berendsen *et al.*, 2012; Baldrian, 2017). Beneficial soil microbes catalyse a variety of soil nutrient cycles, such as C, N, and P (Bahram *et al.*, 2018; Jiao *et al.*, 2018; Pérez-Izquierdo *et al.*, 2019; Zhao *et al.*, 2021). These macro and micro-nutrients are essential for the growth and survival of plants. In contrast, pathogens such as species of *Phytophthora* and *Fusarium* cause serious plant diseases that affect the sustainability of forest ecosystems (Burgess *et al.*, 2017; Elvira-Recuenco *et al.*, 2020; Oliva *et al.*, 2020; Põlme *et al.*, 2020).

Beneficial microbes are vital in the management of soil biogeochemical processes because they are the primary drivers of nutrient cycling and quality improvement (Shi *et al.*, 2019; Zhao *et al.*, 2021). Among them, saprophytic and mycorrhizal fungi play a pivotal role in degrading plant biopolymers and also facilitate nutrient assimilation, improve plant metabolism, and enhance disease tolerance (Voříšková and Baldrian, 2013; Baldrian, 2017; Etalo *et al.*, 2018).

Saprotrophs are primary degraders that contribute significantly to the global carbon cycle (Crowther *et al.*, 2012; Baldrian, 2017). The decomposition of organic matter by these organisms allows inorganic nutrients to be released into the soil, where they are absorbed by plants (Lindahl *et al.*, 2002). Mycorrhizal fungi, on the other hand, mobilise

mineral nutrients such as N and P, in addition to enhancing the mineral nutrient uptake of their hosts (Johnson *et al.*, 2015; Philpott *et al.*, 2018; Tedersoo and Bahram, 2019). The continual recycling of soil nutrients by microbes makes available a consistent supply of these elements in natural ecosystems. But this natural maintenance of soil health does not fully extend to plantation monocultures.

The diversity of soil-associated microbes varies substantially between natural forests and plantations (Chen *et al.*, 2007; Riddell *et al.*, 2019; Byers *et al.*, 2020; Bose *et al.*, 2021). Compared to natural forests, the soil fungal diversity in plantations can be less diverse (Tedersoo *et al.*, 2020; Danielsen *et al.*, 2021). This is predominantly due to anthropogenic disturbances such as a change in land use, continuous monoculture of single tree species, management techniques and harvesting (Luizao *et al.*, 1992; Pietikäinen and Fritze, 1995; Breland and Hansen, 1996; Li *et al.*, 2004; He *et al.*, 2005; He *et al.*, 2009; Pan *et al.*, 2009; Xu *et al.*, 2009; Moora *et al.*, 2014). The consequences of these disturbances impact the aboveground vegetation, soil fungal diversity and soil organic biomass. Hence, plantation forests often negatively impact soil properties by reducing C and N content, changing soil pH, and C decomposition rate (Chazdon, 2003; Rydgren *et al.*, 2004; Rousk *et al.*, 2009; van der Heijden *et al.*, 2015; Zhou *et al.*, 2018).

The loss of microbial diversity in planation soils can be mitigated through adequate post-harvest residue management practises, which replenish soil nutrients through the breakdown of organic residues (Chen and Xu, 2005; Xiong *et al.*, 2021). Furthermore, soil microbes increase the physiochemical and water retention properties of soil, thereby promoting the floral and faunal diversification in forest stands (Blumfield and Xu, 2003; Mathers *et al.*, 2003a; Mathers *et al.*, 2003b; Ali, 2019; Manolis *et al.*, 2019; Bonanomi *et al.*, 2021; Pergola *et al.*, 2022). Thus, adequate post-harvest management can promote the sustainability of plantations.

Forestry enterprises in many parts of the world have initiated research to identify post-harvest strategies that could improve soil health in their plantations (He *et al.*, 2006; Dighton *et al.*, 2012; Klockow *et al.*, 2013; Avera *et al.*, 2020). In this regard, South African forestry companies have initiated various long-term studies including a 'Nutrient Depletion Study' (NDS; Dovey, 2016). The study sites are located in the KwaZulu-Natal Province of

South Africa and were planted with the same *Eucalyptus* genotype. An objective of this long-term research is to describe the fungal microbiome present at the study sites and thus to better understand the efficacy of three post-harvest residue management regimes. These regimes include combinations of retention, removal and fertilisation of the sites. Post-harvest residues were allowed to decompose *in situ* in the 'retained' plots but discarded in the 'removed' plots. Trimmings were removed and inorganic fertilisers were added to 'removed and fertilised' plots.

Previous soil microbial biodiversity studies conducted elsewhere in the world comparing residue management methods, geographic areas, and above-ground vegetation have shown that these factors had a significant impact on the soil microbiome (Shi *et al.*, 2014; Tedersoo *et al.*, 2014; Burgess *et al.*, 2019; Kraut-Cohen *et al.*, 2020; Ammitzboll *et al.*, 2021; Wang *et al.*, 2022). Several post-harvest residue management studies, including the one sampled in this study, have previously been conducted in South Africa (Little *et al.*, 2000; Rolando and Allan, 2004; Dovey *et al.*, 2011; Dovey, 2016; Da Costa Alpoim, 2021; Ramantswana *et al.*, 2021). All previous studies sought to investigate the effects of various management practices on soil nutrients, long-term site sustainability, plant survival and growth, and a variety of other factors. However, none of the previous studies evaluated the impact of different post-harvest residue management regimes on soil-associated microbes. To bridge this knowledge gap, in this study, we describe the fungal microbiome and evaluate the effect of the three post-harvest residue management regimes mentioned above on the soil-associated fungal diversity using a high-throughput sequencing platform. We hypothesised that the soil-associated fungal community (diversity and composition) would (1) vary significantly between the three post-harvest residue management regimes, and (2) be different across the sampling sites.

2. Materials and methods

2.1 Description of study sites

Nutrient Depletion Studies (NDS) were initiated in November 2012 by the Institute for Commercial Forestry Research (ICFR), Pietermaritzburg, in collaboration with major forestry companies in South Africa. One of the aims of this trial was to determine the

efficiency of three post-harvest soil treatment regimes in restoring soil nutrients lost due to continuous planting of monoculture *Eucalyptus* plantations.

The present study focussed on three NDS trial sites located at (i) Windy Hill (29°31'23.0"S 30°34'21.0" E), (ii) Dukuduku (28°18'00.0"S 32°18'55.0" E), and (iii) Clan (29°20'06.0"S 30°27'13.0" E) in the KwaZulu-Natal Province of South Africa (Fig. 1). These three trial sites were established in (i) November 2012 (ii), April 2013, and (iii) January 2015, respectively. The three sites are located in two South African biomes: grassland (Clan and Windy Hill) and Indian Ocean coastal (Dukuduku) (Dovey, 2016). All three trial sites measured 28 × 27 m and were planted to a single genotype of a hybrid between *Eucalyptus grandis* and *E. urophylla*. Each trial plot was divided into 12 sub-plots, measuring 5 × 5 m, separated by buffer planting zones (Fig. 1). Four sub-plots were dedicated to each of the three post-harvest soil treatment regimes. These were categorized as 'retained', 'removed' and 'removed and fertilized' (Fig. 1). In the 'retained' plots, post-harvest residues were retained by leaving all leaves and branches in the plot. In the 'removed' plots all post-harvest trimmings were removed from the plot. In the 'removed and fertilized' plots the trimmings were removed followed by the addition of inorganic fertilizers once each year to compensate for the nutrient deficiency and lateral movement of nutrients. The chemical composition of the plant trimmings removed from the plot at each study site was used to determine the inorganic constituents of the fertiliser and their proportions. Consequently, the composition of this fertilizer varied between years and study sites. To simulate the impact of multiple rotations on soil nutrients, trees were planted at very narrow spacing (1 m apart) and stems were felled at least once a year and allowed to coppice.

2.2 Collection of soil samples

In February 2019, 36 soil samples were collected at every NDS site (3 treatments × 4 sub-plots per treatment × 3 soil samples per sub-plot). Soil samples were collected arbitrarily around the bases of trees after removing the plant litter and 2-3 cm of the topsoil, at a linear distance of at least 2 m away from each other and the sub-plot boundary. Root pieces from each soil sample were carefully separated and discarded. Then, the soil samples

were stored individually in sterile polypropylene bags. A total of 108 soil samples were collected from the three trial sites (Fig. 1).

2.3 Extraction of environmental DNA

All soil samples were dried at room temperature (21-23 °C) for two weeks. Subsequently, about 20 g of each soil sample was pulverized using a Retsch grinding jar attached to a Qiagen TissueLyser II for 2 min at 20 frequency/sec. Between the pulverization steps, the grinding jars were surface sterilized with 4M hydrochloric acid and 4% (v/v) sodium hypochlorite solution and rinsed using sterile deionised water. All the pulverized soil samples were stored at -20 °C until DNA could be extracted.

Environmental DNA (eDNA) was extracted from 0.5 g of each soil sample using the Qiagen DNeasy PowerSoil Kit (Carlsbad, CA) following the manufacturer's protocols. After extraction, all eDNA samples were stored at -20 °C until the fungal amplicon library preparation.

2.4 Preparation of fungal amplicon library

For each eDNA sample, the amplicon library was prepared by performing two consecutive PCRs. In the first PCR, the complete Internal Transcribed Spacer (ITS) of the nuclear rDNA was amplified using the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990; Gardes and Bruns, 1993). Each ITS primer used in this PCR was labelled with a 'tag' and an 'overlap' (5' overlap-tag-ITS1F 3' and 5' ITS4-tag-overlap 3'). The total volume of each amplification was 12.5 µL which included 0.5 µL of eDNA, 6.25 µL of GoTaq Colourless Master Mix (Promega, Germany), 0.25 µL (10 µmol/L) of each primer, and 5.25 µL of PCR grade water. PCR conditions were 3:00 min at 95 °C, followed by 32 cycles of 27 sec at 94 °C, 1:00 min at 57 °C, 1:30 min at 72°C, and final elongation for 7:00 min at 72°C. After amplification, products were visualised using agarose gel electrophoresis. Thereafter, 5 µL of this PCR product was cleaned using ExoSAP-IT PCR Product Clean-up Reagent (Thermo Fisher Scientific, USA) following the manufacturer's

protocols. This cleaned PCR product was used as the template for the second round of amplification.

In the second PCR, the samples were labelled with 8 bp long 'indexes' (5' P5 tail-index-overlap 3' and 5' overlap-index-P7 tail 3') for post-sequence identification. The total volume of each amplification was 25 µL, which included 5 µL of cleaned template DNA from the first amplification, 12.5 µL of GoTaq Colourless Master Mix (Promega, Germany), 0.5 µL (10 µmol/L) of each primer, and 6.5 µL of PCR grade water. PCR conditions were 3:00 min at 95 °C, followed by 5 cycles of 27 sec at 94 °C, 1:00 min at 57 °C, 1:30 min at 72°C, and final elongation for 7:00 min at 72°C. The PCR products from this amplification step were visualised using agarose gel electrophoresis.

2.5 Pooling of amplicon libraries

To determine the relative concentrations of the PCR products, the gel images from the second round of amplification were analysed using the software ImageJ v1.52q (Schneider *et al.*, 2012). Based on their concentrations, the samples were pooled into groups. These pooled groups were cleaned using Agencourt AMPure XP PCR purification beads (Beckman Coulter Genomics, USA) following the manufacturer's protocol and visualized using agarose gel electrophoresis. These steps were continued until the final pooling phase, in which all of the groups were combined into a single unit.

2.6 Sequencing of amplicon library

The fungal amplicon library was quality controlled with DNA High Sensitivity DNA Kit on Bioanalyzer (Agilent) and quantified on Qubit 2.0 Fluorometer (ThermoFisher Scientific with ds HS Assay Kit). Amplicon sequencing was performed on an Illumina MiSeq (Illumina Inc., San Diego, CA, USA) using the MiSeq® Reagent Kit v3 Chemistry (2 x 300 bp paired-end) at the Genomics Service Unit (LMU Biocenter, Planegg-Martinsried, Germany).

235 *2.7 Analyses of high-throughput sequence data*

236 The Illumina MiSeq data were de-multiplexed using the Quantitative Insights into
237 Microbial Ecology 1 (QIIME1) v 1.5.8 (Caporaso *et al.*, 2010) based on forward and
238 reverse tags and indexes. Primers, indexes, tags, and adapters were removed at this step.
239 Only forward reads were used for further analyses due to the short-read length, which
240 prevented the merging of forward and reverse reads. The retained forward reads were
241 deposited in the NCBI Sequence Read Archive (https://submit.ncbi.nlm.nih.gov/subs/sra/)
242 under the accession number PRJNA714498.

243 Subsequent analysis of high-throughput sequence data was done using the plugins
244 and software available through QIIME2 v2020.8 (Bolyen *et al.*, 2019). Denoising,
245 trimming, deletion of chimaeras and singletons, and filtering of the reads were performed
246 using the plugin 'q2-dada2' (Callahan *et al.*, 2016). *De novo* assembly of the reads into
247 Operational Taxonomic Units (OTUs) was done at a 98% sequence similarity using the
248 plugin 'q2-vsearch' (Rognes *et al.*, 2016) and the 'qiime feature-classifier' plugin
249 (Bokulich *et al.*, 2018) was used for assigning taxonomy to the OTUs. In this step of the
250 analysis, UNITE fungal ITS database v8.2 (Abarenkov *et al.*, 2020) was used as the
251 reference. The identity of those OTUs that could not be resolved using the UNITE
252 reference database was reanalysed using the online 'blastn' algorithm available through the
253 NCBI GenBank (Altschul *et al.*, 1990).

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255 *2.8 Functional characterization of the fungal community*

256 To explore the function of the fungal community, the FUNGuild v1.1 (Nguyen *et al.*,
257 2016) was used to characterize the trophic mode and functional groupings (guilds) for all
258 three post-harvest soil treatment regimes across three sample locations. In this analysis,
259 two confidence levels were regarded as reliable: 'highly probable' and 'probable'. All
260 OTUs that did not match any taxa in the database were designated as 'unassigned'.

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2.9 Statistical analyses

The taxonomic composition of soil fungi was visualised using a heat tree generated with the *MetacodeR* package in the R software (Foster *et al.*, 2017; R Core Team, 2021).

To analyse the soil fungal diversity, species richness and Shannon and Simpson diversity indexes were calculated. Species richness was calculated as the number of different OTU per sample. Shannon index consideres species richness and abundance using a logarithmic scale (1) and the Simpson index measures dominance, where common species have a lot of weight compared to rare species (2).

(1)

$$H = -\sum_{i=1}^{s} p_i \times \ln p_i$$

i = each species

s = total number of species

p_i = relative abundance of each species in the community

(2)

$$D_s = 1 - \sum \left(\frac{n}{N}\right)^2$$

n = number of individuals for each species

N = total number of all individuals

To explore differences in species richness and diversity indexes among the post-harvest soil treatment regimes and the sites, ANOVAs were used. 'Post-harvest soil treatment regimes', 'sites' and their interaction were included as explanatory variables in the models. In all cases, model validity was checked. Tukey's Honest Significant Difference (HSD) post hoc test was applied to do pairwise comparisons of the means. The *agricolae* package of R software (R Core Team, 2021) was used to analyse soil fungal diversity.

Soil fungal community composition for the different post-harvest soil treatment regimes and sites was visualized using a Principal Coordinate Analysis (PCoA). PCoA was performed on an abundance matrix applying Bray-Curtis dissimilarity. To assess whether community composition differed statistically among different post-harvest soil treatment

regimes and/or sites, a permutational multivariate analysis of variance (PERMANOVA) was used. Community composition varied to a greater extent between study sites than among post-harvest soil treatment regimes. Therefore, the influence of post-harvest soil treatment regimes on fungal communities within each site was analysed. In cases where the PCoA failed to show a pattern but the PERMANOVA was statistically different and the value of R was not very large, a permutational multivariate analysis of dispersion was used (PERMDISP, Anderson, 2006; Anderson and Walsh, 2013). PERMDISP analyses the dispersion of the data to assess whether the differences are differences in dispersion and not differences in community structure. The *vegan* package of the R software (Oksanen *et al.*, 2018; R Core Team, 2021) was used to analyse the community composition.

The soil fungal community composition was also illustrated using differential heat trees. Heat trees represent the significant differences in the abundance of each fungal taxon among the three post-harvest soil treatment regimes and the three sites. A Wilcoxon Rank Sum test was used, and the resulting p-values were corrected for multiple comparisons using the false discovery rate (FDR). The *MetacodeR* package in the R software (Foster *et al.*, 2017; R Core Team, 2021) was used to construct the differential heat trees.

3. Results

3.1 Soil fungi associated with the rhizosphere

A total of 4,206,971 raw reads emerged from the Illumina high-throughput sequencing of 108 eDNA samples. After filtering these raw reads using the 'q2-dada2' pipeline, 3,714,437 (88.3 %) reads were used for downstream analyses. A total of 607 fungal OTUs were identified after *de novo* assembly and taxonomic assignment of these filtered reads. The majority of the fungal OTUs were represented by Ascomycota (56.01 %), followed by Basidiomycota (36.3 %). The remaining 7.7 % of the OTUs were identified as fungi representing the phyla Calcarisporiellomycota, Chytridiomycota, Entorrhizomycota, Glomeromycota, Kickxellomycota, Mortierellomycota, Mucoromycota, Rozellomycota, and Zoopagomycota (Fig. 2, Table S1).

Among the sites, the highest number of OTUs were detected from Clan (392) followed by Dukuduku (355) and Windy Hill (347). A substantial portion of the OTUs

(28.3 %) was shared between samples from the three collection sites (Fig. S1A, Table S1). Compared to Windy Hill and Dukuduku, Clan had the highest percentage (19.3 %) of exclusive fungal taxa (Fig. S1A). The highest number of shared fungal OTUs (11.4 %) was between Clan and Windy Hill (Fig. S1A).

Among the three post-harvest soil treatment regimes, the richness of OTUs was greatest in plots where the post-harvest residues were retained (444) followed by plots where post-harvest residues were removed and fertiliser was added (413) and plots where post-harvest residues were removed (405). A substantial portion of fungal OTUs (44.5 %) was shared between the three soil treatment regimes (Fig. S1B). The highest number of exclusive fungal OTUs (14.5 %) was detected in the 'retained' plots followed by 'removed and fertilized' and 'removed' (Fig. S1B, Table S1).

At each sampling site, a substantial number of fungal taxa was shared between the three post-harvest residue management regimes (Fig. S1C, D and E). The 'retained' plots at Clan and Dukuduku had the highest number of exclusive fungal OTUs (Fig. S1C, D, and E, Table S1). This was not observed for Windy Hill.

Among the OTUs detected from the Ascomycota, the class Dothideomycetes included pathogens of *Eucalyptus*. These were from the families Botryosphaeriaceae, Mycosphaerellaceae, and Teratosphaeriaceae. A majority of these pathogens were from genera such as *Devriesia, Diplodia*, *Lasodiplodia*, *Pseudocercospora*, *Ramularia*, *Teratosphaeria*, and *Zasmidium* (Fig. 2, Table S1).

The OTUs representing Basidiomycota and Glomeromycota included fungi that form symbiotic relationships with *Eucalyptus*. All ectomycorrhizal fungi found in soils collected from all three post-harvest regimes were assigned to the class Agaricomycetes whereas the arbuscular mycorrhizal taxa were from the Glomeromycetes. Potential ectomycorrhizal fungi were from the genera *Amanita*, *Cortinarius*, *Pisolithus*, *Laccaria*, *Scleroderma*, *Tomentella*, and *Tricholoma*. OTUs of ectomycorrhizal fungi from the Thelephoraceae and Russulales were also detected in soils, but their identities could not be determined to the genus or species level (Table S1). Arbuscular mycorrhizal fungi were from the families Gigasporaceae or Glomeraceae. Gigasporaceae was represented by genera such as *Cetraspora* and *Gigaspora* (Table S1). All fungal OTUs detected from the family

353 Glomeraceae remained unclassified. Simultaneously, fungi known to form symbiotic
354 associations with other plant species, such as *Lepiota*, *Macrolepiota*, *Inocybe*, and others,
355 were also found in the soils.

356 Various lineages of saprophytic fungi representing Ascomycota, Basidiomycota,
357 Mortierellomycota, and Mucoromycota were detected in all soil types. Saprophytic
358 Ascomycota lineages detected from soils were from the classes Dothideomycetes,
359 Eurotiomycetes, Pezizomycetes, and Sordariomycetes such as *Annulohypoxylon,*
360 *Aspergillus*, *Chaetomium*, *Penicillium*, *Ruhlandiella*, *Trichoderma*, *Westerdykella*, and
361 *Xylaria*. The bulk of saprophytic Basidiomycota was from the class Agaricomycetes, such
362 as *Clitopilus*, *Coniophora*, *Conocybe*, *Coprinellus*, *Geastrum*, *Hyphoderma*, *Lepista*,
363 *Marasmius*, *Mycena*, *Phanerochaete*, and *Tomentella*, with a few from the
364 Tremellomycetes. Mortierellomycota was represented by Mortierellales and Endogonales.
365 Saprophytes from Mucoromycota were from Mucorales and Umbelopsidales.

366

367 *3.2. Functional characterization of the fungal community*

368 FUNGuild made it possible to determine the tropic mode and guild for a majority of
369 OTUs (445) (Table S1). The confidence level for a majority of OTUs was "probable" (323),
370 followed by "highly probable" (64) and "possible" (52) (Table S1). Seven trophic levels
371 were detected among the OTUs (Table S1, Fig. S2). These were pathotroph, pathotroph-
372 saprotroph, pathotroph-saprotroph-symbiotroph, pathotroph-symbiotroph, saprotroph,
373 saprotroph-symbiotroph, and symbiotroph (Fig. S2).

374 At Clan and Windy Hill, plots where the post-harvest residue was retained had a
375 higher prevalence of saprotrophs and symbiotrophs. "Removed and fertilised' plots also
376 had a higher prevalence of symbiotrophs along with pathotrophs. The plots from which
377 post-harvest residues were removed were poor in both beneficial and detrimental fungi
378 (Fig. S2). However, this trend was not found at Dukuduku, where "retained' and "removed'
379 plots both had a comparable number of saprotrophs, symbiotrophs and pathotrophs (Fig.
380 S2). In contrast, the percentages of both beneficial and detrimental fungi were lower in the
381 'removed and fertilised' plot (Fig. S2).

382 *3.3 Effect of soil treatments on the diversity of soil fungi*

383 The species richness of soil fungi was substantially different between the three post-
384 harvest soil treatment regimes ($P < 0.01$; Table 1). Compared to 'removed' plots, species
385 richness was higher in 'retained' plots ($P < 0.01$). Shannon and Simpson's indexes were
386 influenced by the different sites ($P < 0.001$; Table 1). All diversity indexes assessed
387 (richness and Shannon and Simpson indexes) were significantly influenced by the
388 interaction between post-harvest soil treatments and sites (Table 1 and Fig. S3).
389
390 *3.4 Effect of sample location and soil treatments on fungal community composition*
391 The PCoA plot illustrated that fungal community composition was different between
392 the three study sites (Fig. 3A). PERMANOVA confirmed that the different study sites and
393 post-harvest soil treatment regimes influenced the community composition of soil fungi
394 (Table 2). For the within-site analyses, the PCoAs and PERMANOVASs showed that
395 community composition at the Clan and Windy Hill sites was significantly different
396 between post-harvest soil treatment regimes (Fig. 3B and D, respectively; Table 2). For the
397 Dukuduku site, the PCoA did not provide a pattern (Fig. 3C) but the PERMANOVA
398 showed that community composition was significantly different between post-harvest soil
399 treatment regimes with a low percentage of statistical variance (low r^2, Table 2). In this
400 situation, PERMDIP demonstrated that the differences shown by the PERMANOVA at the
401 Dukuduku site (Table 2) were due to significant differences in the dispersion of the samples
402 ($F = 6.9187$; $P < 0.01$) and not by the fungal community composition.
403 The differential heat tree up to the order level showed the abundance of different
404 fungal taxa across three post-harvest soil treatment regimes (Fig. 4) and between three
405 different sites (Fig. S4). As expected, compared to 'removed' plots, soils from the
406 'retained' and 'removed and fertilized' treatments had a higher abundance of fungi
407 representing the majority of fungal phyla detected in this study, such as Ascomycota,
408 Basidiomycota, Calcarisporiellomycota, Mortierellomycota, Mucoromycota, and
409 Rozellomycota (Fig. 4). Compared to Clan and Dukuduku, Windy Hill had a much higher
410 abundance of potentially pathogenic fungi from the families Botryosphaeriaceae,
411 Diaporthaceae, Mycosphaerellaceae, Nectriaceae, and Teratosphaeriaceae, whereas Clan
412 had the greatest number of fungi from the Ophiostomatales (Table S1).

4. Discussion

The community composition and diversity of soil fungi associated with three post-harvest residue management regimes were shown in this study to be significantly different. Most importantly, the analyses of high-throughput sequencing data showed that (1) the fungal species diversity (species richness, and Shannon and Simpson diversity indexes) was influenced by the interaction between the post-harvest treatments and the site. Specifically, the fungal species richness differed substantially among the three post-harvest soil treatment regimes, with the plots retaining post-harvest trimmings having the highest species richness in comparison with the "removed" plots, and (2) the fungal community composition was significantly influenced by the geographic location of the sampling sites.

4.1 Soil fungi associated with the rhizosphere

At least 11 fungal phyla were detected from soil samples collected from three sampling sites. Irrespective of treatment, the majority of fungal OTUs detected from the soil were Ascomycota and Basidiomycota (92.3 %) and the remaining nine fungal phyla were rare. Various previous studies of fungal diversity using high-throughput sequencing platforms have reported a similar soil-associated community composition (Tedersoo *et al.*, 2014; Toju *et al.*, 2014; Urbina *et al.*, 2016; Jimu *et al.*, 2018). In the present study, fungi in the Ascomycota and Basidiomycota included those known to be pathogens and symbionts of *Eucalyptus* (Díez, 2005; Chen *et al.*, 2007; Carrenho *et al.*, 2008; Ducousso *et al.*, 2012; Wingfield *et al.*, 2015; Jimu *et al.*, 2018). This was not surprising given that the diversity of soil microorganisms usually represents such a range and is reliant on the plants with which they interact physiologically (Schlaeppi and Bulgarelli, 2015; Baldrian, 2017).

At each of the three sampling sites, a majority of the OTUs represented Ascomycota. This is consistent with prior studies that considered fungal diversity in forestry settings, where the Ascomycota diversity was always higher than that of other fungal phyla (Tedersoo *et al.*, 2014; Yang *et al.*, 2017; Jimu *et al.*, 2018; Li *et al.*, 2019; Tomao *et al.*, 2020). A substantial portion of these fungi was associated with the plots where the post-harvest residues had been retained. This group of fungi includes saprophytes, as well as

443 those that are pathogenic to various species of *Eucalyptus* (Wingfield *et al.*, 2008; Schoch
444 *et al.*, 2009; Kemler *et al.*, 2013; Tomao *et al.*, 2020). Saprophytic fungal OTUs resided in
445 the Dothideomycetes, Eurotiomycetes, Orbiliomycetes, Sordariomycetes, and
446 Pezizomycetes. The presence of these fungi in the plantation soils is likely associated with
447 their role as primary degraders of organic matter (Baldrian, 2017; Tomao *et al.*, 2020).

448 Several previously identified stem and foliar pathogens of *Eucalyptus* from the class
449 Dothideomycetes (Botryosphaeriaceae, Mycosphaerellaceae, and Teratosphaeriaceae),
450 such as *Devriesia, Ramularia, Teratosphaeria* and few others were detected from soils.
451 These foliar pathogens in the soil likely originated from decomposing *Eucalyptus* leaves
452 and branches. At Clan and Windy Hill, the total number of reads detected from
453 Dothideomycetes was higher in the removed and fertilised plots than in the other two
454 regimes, whereas the inverse was true at the study site in Dukuduku. This might be
455 attributed to changes in edaphic variables such as pH and nutrients between the soils, which
456 have previously been linked to effects on plant pathogens in the soil (Zheng *et al.*, 2017;
457 Castaño *et al.*, 2019). Similarly, plantation soils may serve as one of the sources of
458 inoculum for some of the pathogens of *Eucalyptus*. However, there is no evidence from
459 this study to support the aforementioned hypotheses.

460 At least 220 OTUs from Basidiomycota, representing 32 orders, were detected in the
461 soils collected from the study sites. This diversity included various taxa of Agaricomycetes
462 that are known to form an ectomycorrhizal relationship with *Eucalyptus* species including,
463 for example, *Amanita*, *Cortinarius*, *Pisolithus*, *Laccaria*, *Scleroderma*, *Tomentella*,
464 *Tricholoma*, and fungi in the Thelephoraceae, and Russulales (Malajczuk *et al.*, 1982;
465 Thomson *et al.*, 1994; Keane, 2000; Díez, 2005; Ducousso *et al.*, 2012; Kluthe *et al.*, 2016).
466 As previously documented in Europe and Africa, ectomycorrhizal fungi in the genera
467 *Laccaria*, *Tricholoma*, and *Pisolithus* were most likely introduced with *Eucalyptus* from
468 Australia (Díez, 2005; Kluthe *et al.*, 2016; Jimu *et al.*, 2018). Many of the ectomycorrhizal
469 species identified in this study form basidiocarps and can also co-exist as saprobes, such
470 as *Cortinarius*, *Tricholoma*, *Laccaria* and many more. As a result, retaining post-harvest
471 trimmings in plantations could provide a substrate for these fungi to grow, hence increasing
472 mycorrhizal biodiversity in plantation soil.

4.2 Influence of sample location and soil treatments on the community diversity

Fungal diversity was affected by the interaction between the soil treatments and the sites. This was to be expected, as the geographic location is an important factor that influences the community diversity of soil-associated fungi and fungi-like organisms (Shi *et al.*, 2014; Tedersoo *et al.*, 2014; Burgess *et al.*, 2019; Bose *et al.*, 2021). The richness associated with plots retaining post-harvest residues was substantially greater than those where residue was removed. This is most likely because post-harvest residues act as the substrate for these fungi that decompose these organic residues (Uroz *et al.*, 2016; Baldrian, 2017). As a result, retaining post-harvest residues would be a cost-effective and long-term strategy to increase the biodiversity of soil fungi in plantations.

Previous studies have shown that retaining post-harvest residues has the potential to increase plant biomass and improve nutrient cycles in plantation soils (Mathers *et al.*, 2003a; Mendham *et al.*, 2003; Kumaraswamy *et al.*, 2014; Mendham *et al.*, 2014; Zhu *et al.*, 2020). Soil fungi can contribute to this effect by breaking down the organic residues over time, which in turn improve the nutrient content of the soil. In the future, it would be interesting to see if the decomposition of plant trimmings substantially improves the nutrient content of the soil retaining post-harvest trimmings compared to removed plots.

4.3 Influence of sample location and soil treatments on the community composition

The soil community composition varied significantly between the NDS study sites. This was not surprising given the disparate geographic locations of the sites. Study sites at Clan and Windy Hill are in the grassland biome, whereas Dukuduku is in the Indian Ocean coastal biome (Dovey, 2016). Abiotic factors such as climate and soil properties are known to vary significantly along the latitudinal gradient, and biomes influence the community of soil-associated microbes (Shi *et al.*, 2014; Tedersoo *et al.*, 2014; Burgess *et al.*, 2019; Bose *et al.*, 2021). Simultaneously, the efficacy of the three soil treatments at each study site was also regulated by these abiotic properties, which could be evaluated by the difference in soil fungal biodiversity.

Fungi were more abundant in the soils where post-harvest residues had been retained or where they had been removed but then fertilised, in comparison to the plots where the residues had been removed. This higher abundance of fungi linked with these two post-

harvest soil treatment regimes included possible beneficial and harmful fungi along with many others. These results imply that the application of fertiliser can enhance the biodiversity of soil fungi. However, the regular application of fertilisers to forest soil would be impractical and uneconomical (Dias and Arroja, 2012; Pahalvi *et al.*, 2021; Vasco *et al.*, 2021). Retaining post-harvest residues in forest plots would be preferable and be an environmentally benign approach to retaining soil nutrients and microbes in plantations.

5. Conclusions

The high-throughput sequence data from this study revealed that the fungal community differed substantially between the three post-harvest soil treatment regimes and across sampling sites. FUNGuild analysis showed that the plots at all sampling sites where post-harvest residues had been retained included a diverse range of beneficial fungi, such as saprotrophs and symbiotrophs. Applying fertiliser to the soil where these residues had been removed, increased the number of symbiotrophs as well as pathotrophs, the latter of which could be harmful to tree health. Compared to fertilization, preserving post-harvest residues would be economically and ecologically acceptable and would not require regular reapplications. This is because the decomposition of these residues would provide a steady source of nutrients to the trees over time. Furthermore, and as found in studies conducted elsewhere in the world, the elevated level of beneficial soil microbes associated with retained post-harvest residues, could also improve the physical, chemical, and water retention capabilities of soil as well as the understory vegetation. This would, in turn, augment the sustainability of plantations over time and consecutive rotations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The high-throughput sequence data generated in this study has been submitted to the NCBI Sequence Read Archive (https://submit.ncbi.nlm.nih.gov/subs/sra/) under the accession number PRJNA714498.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at (web link will be added after the final decision)

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Figure legends

Fig. 1. An example site layout of the Nutrient Depletion Study plot located at Dukuduku, KwaZulu-Natal Province of South Africa. At each site, the plot was equally divided into 12 sub-plots separated by buffer planting zones, indicated in yellow. Four of these sub-plots were assigned to each of the three post-harvest residue management regimes: retained, removed, and removed and fertilised. In the "retained" plots, post-harvest residues were maintained by leaving all leaves and branches in the plot, whereas in the "removed" plots, all post-harvest trimmings were removed from the plot. In the "removed and fertilized" plots, the trimmings were removed first, followed by the addition of inorganic fertiliser.

Fig. 2. The taxonomic composition of all fungi associated with the soil from three different post-harvest residue management regimes. The heat tree represents a fungal community structure as a taxonomic hierarchy up to the ordinal level. Node and edge size are proportional to the number of OTUs within each taxon, and colour represents taxon abundance (sum of reads).

Fig. 3. Principal Coordinates Analysis (PCoA) of soil fungal community composition associated with three post-harvest residue management regimes (retained, removed, removed and fertilized) at three study sites. (A) the three sites combined; (B) Clan; (C) Dukuduku; and (D) Windy Hill.

Fig. 4. Differential heat tree up to the order level, illustrating the effect of three post-harvest soil treatment regimens (i.e., retained, removed, and removed and fertilized) on the abundance of soil-associated fungal diversity. Node and edge sizes are proportional to the different number of OTUs between the groups for each taxon and colour represent the log of the ratio of median abundance between the groups for each taxon. When a taxon has more counts in samples from groups on the right side of the graph (retained and removed),

it is coloured green. And, when a taxon has more counts in samples from groups on the upper side of the graph (removed and removed and fertilised), it is coloured brown.

Table legends

Table 1. Results of the ANOVA for the analysis of the influence of the study sites and treatments on soil fungal diversity showing degrees of freedom (*df*), *F* values, and significance levels for each fixed factor.

Table 2. Results of the PERMANOVA for the analysis of the influence of the study sites and treatments on soil fungal community composition showing degrees of freedom (*df*), *F* values, r^2 and significance levels for (A) all the sites together, (B) Clan, (C) Dukuduku and (D) Windy Hill.

Supplementary Figure legends

Fig. S1. Fungal OTU distribution across (A) three sampling sites, (B) three post-harvest residue management regimes combined, and (C) Clan, (D) Windy Hill, and (E) Dukuduku.

Fig. S2. Bar plots summarising the tropic levels of fungal OTUs associated with three post-harvest soil treatment regimes (retained, removed, and removed and fertilized) across three sampling sites (Clan, Windy Hill, and Dukuduku).

Fig. S3. Box plots comparing OTU richness and diversity indices among the three post-harvest residue management regimes and the sampling sites. (A) species richness, (B) Shannon, and (C) Simpson diversity indices of soil fungal communities associated with three post-harvest soil treatment regimes, retained' (RT), 'removed' (RM), and 'removed and fertilised' (RF) in three different sites (Clan, Dukuduku and Windy Hill) . Different letters indicate significant differences ($P<0.05$).

Fig. S4. Differential heat tree up to the order level, illustrating the effect of three sampling sites (i.e., Clan, Windy Hill, and Dukuduku) on the abundance of soil-associated fungal diversity. Node and edge sizes are proportional to the different number of OTUs between

the groups for each taxon and colour represent the log of the ratio of median abundance between the groups for each taxon. When a taxon has more counts in samples from groups on the right side of the graph (Clan and Windy Hill), it is coloured green. And, when a taxon has more counts in samples from groups on the upper side of the graph (Windy Hill and Dukuduku), it is coloured brown.

Supplementary table legend

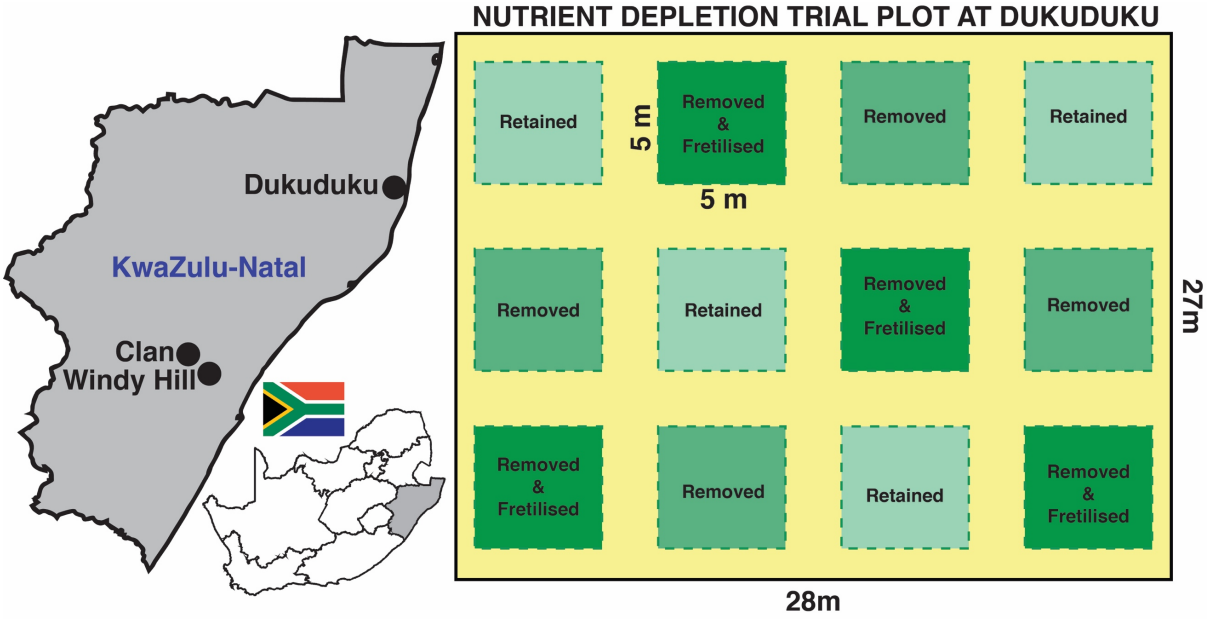
Table S1. List of fungal OTUs detected from 108 soil samples collected from the three Nutrient Depletion Trial sites at Clan, Windy Hill, and Dukuduku, together with their associated FUNGuild annotation.

Table 1
Results of the ANOVA for the analysis of the influence of the study sites and treatments on soil fungal diversity showing degrees of freedom (*df*), *F* values, and significance levels for each fixed factor.

Diversity indexes	**Fixed factors**	***df***	***F* value**	***P* value**
Species richness	Site	2	1.03	0.359
	Treatments	2	5.64	< 0.01
	S × T	4	3.592	< 0.01
Shannon index	Site	2	36.95	< 0.001
	Treatments	2	0.075	0.928
	S × T	4	11.22	< 0.001
Simpson index	Site	2	28.23	< 0.001
	Treatments	2	1.80	0.17
	S × T	4	7.83	< 0.001

Table 2
Results of the PERMANOVA for the analysis of the influence of the study sites and treatments on soil fungal community composition showing degrees of freedom (*df*), *F* values, r^2 and significance levels for (A) all the sites together, (B) Clan, (C) Dukuduku, and (D) Windy Hill.

	Sites	**Explanatory variable**	***df***	***F* value**	**r^2**	***P* value**
(A)	All sites	Site	2	44.51	0.399	< 0.001
		Treatments	2	5.81	0.052	< 0.001
		S × T	4	5.79	0.104	< 0.001
(B)	Clan	Treatments	2	5.0643	0.23485	< 0.001
(C)	Dukuduku	Treatments	2	4.4094	0.21088	< 0.001
(D)	Windy Hill	Treatments	2	7.5803	0.31479	< 0.001



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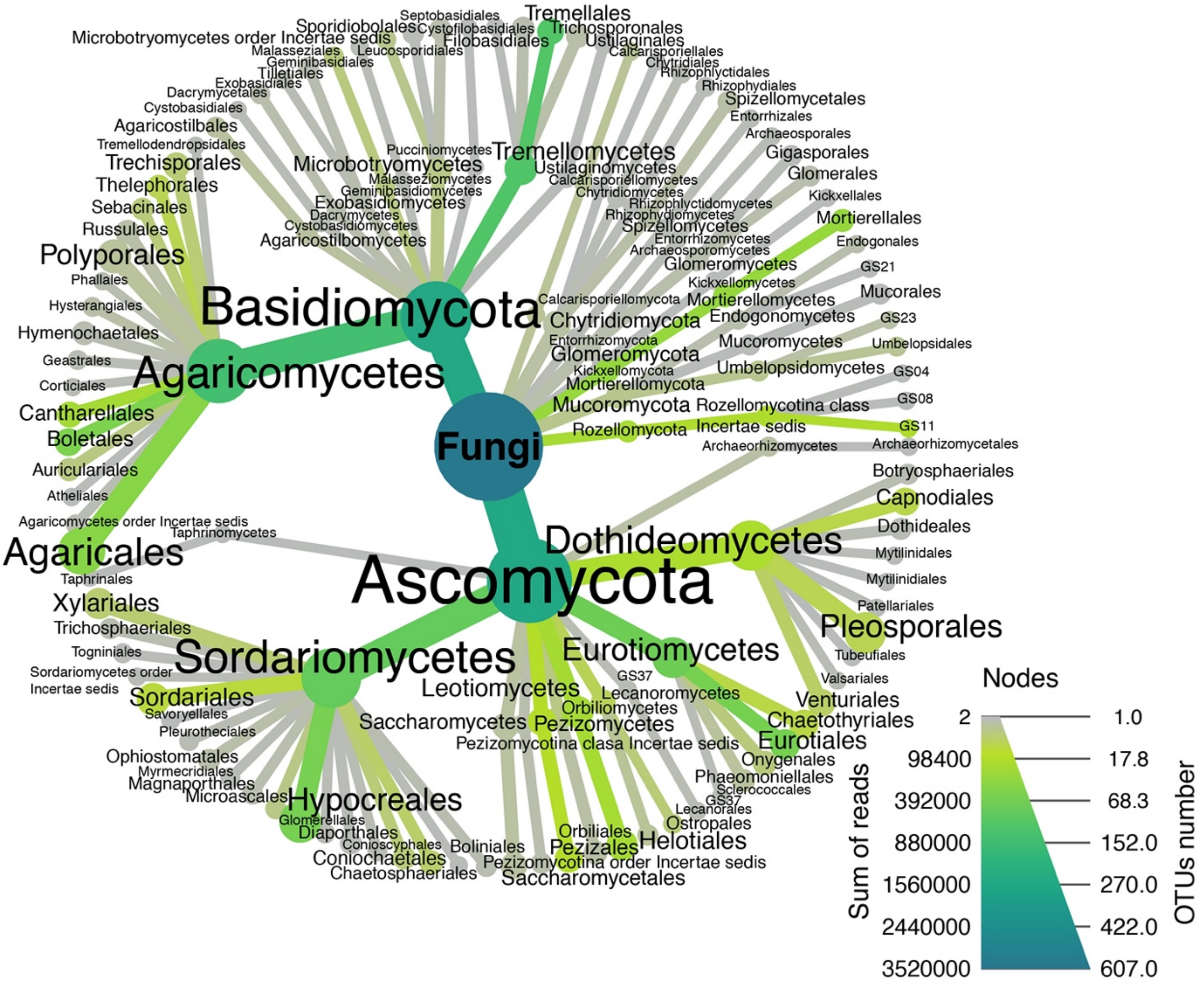

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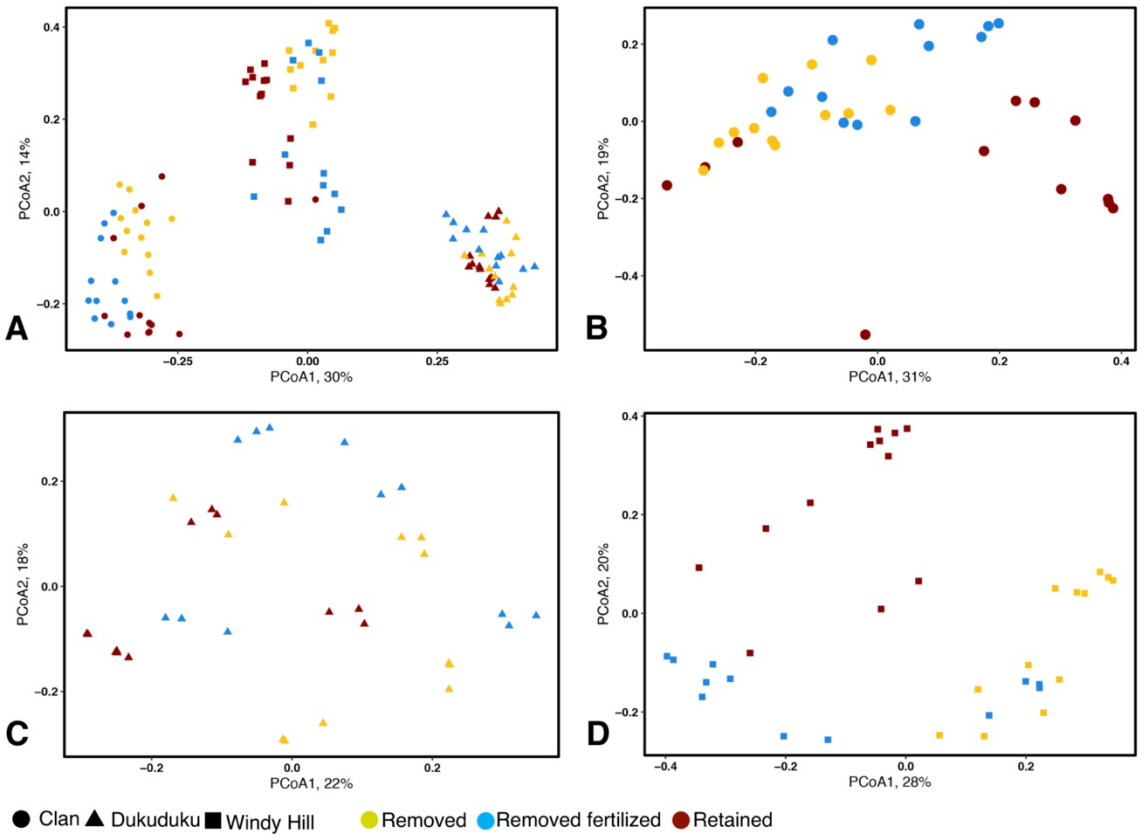

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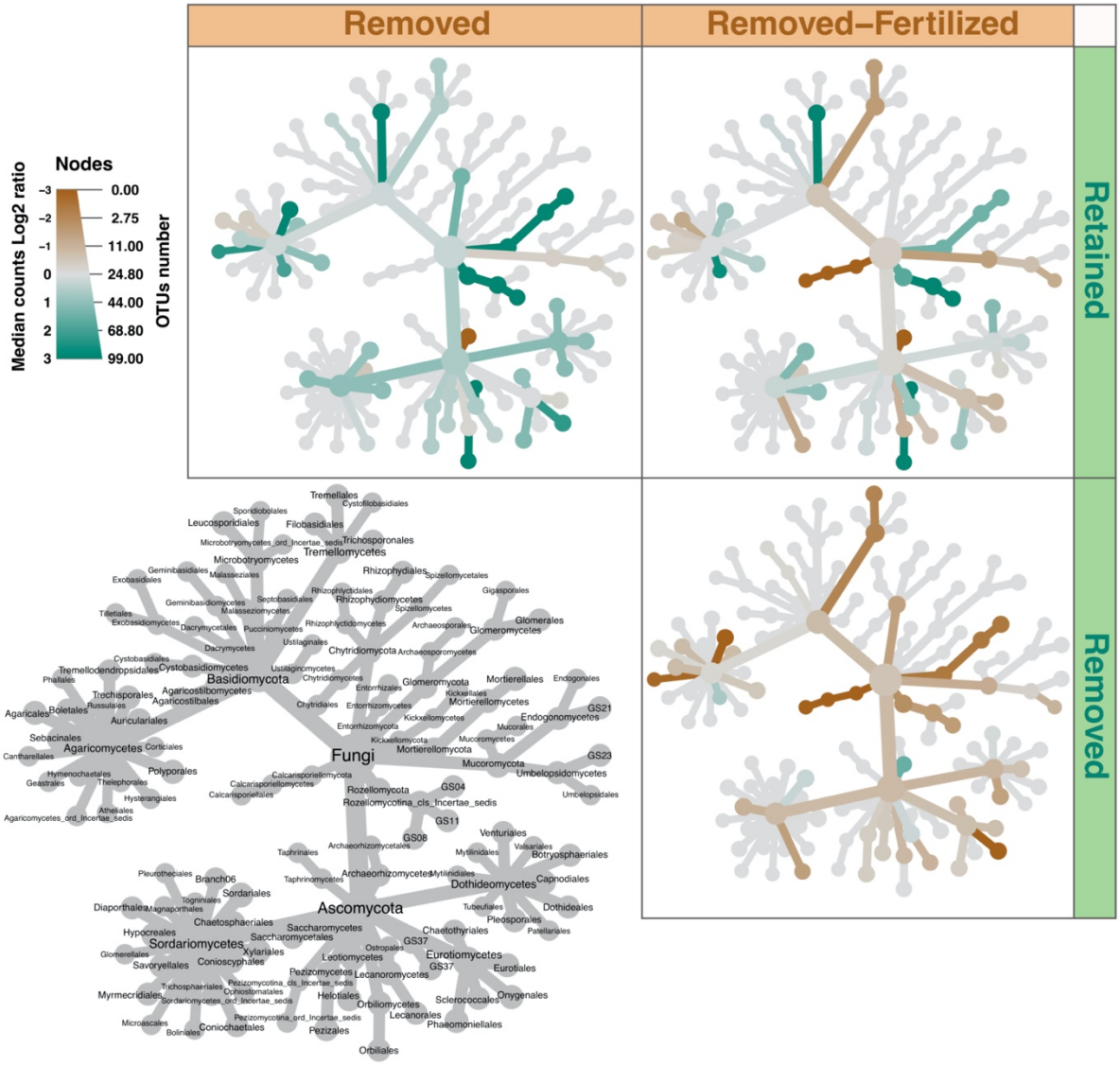

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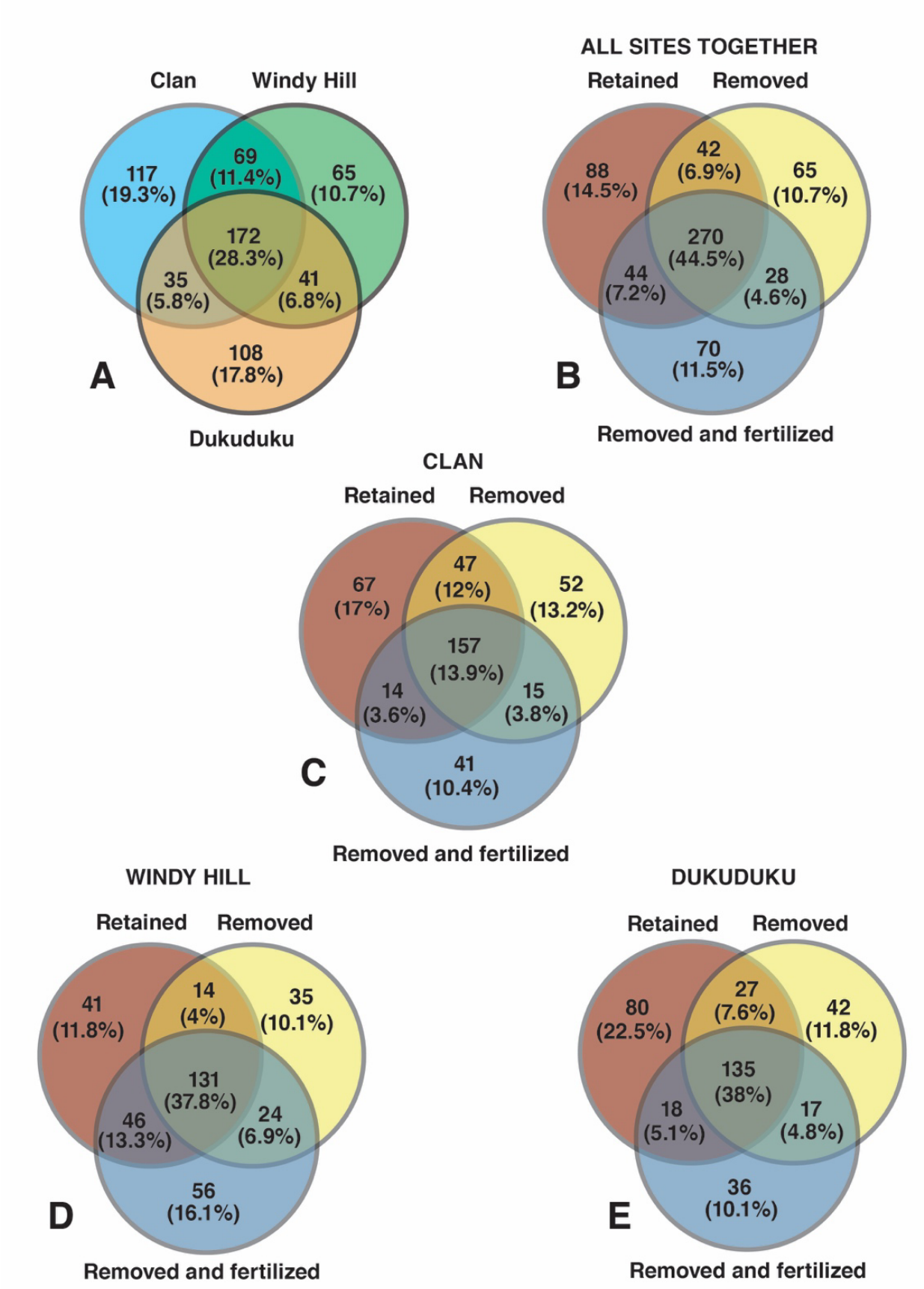

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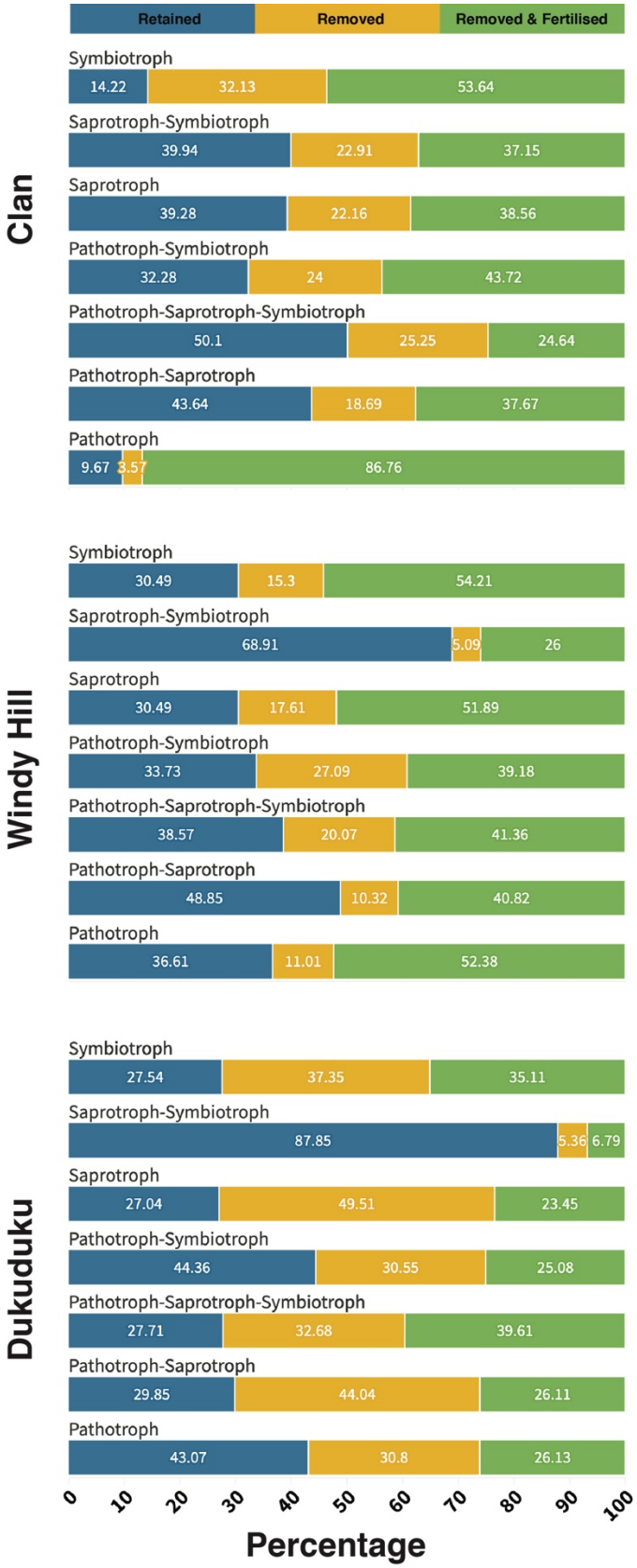

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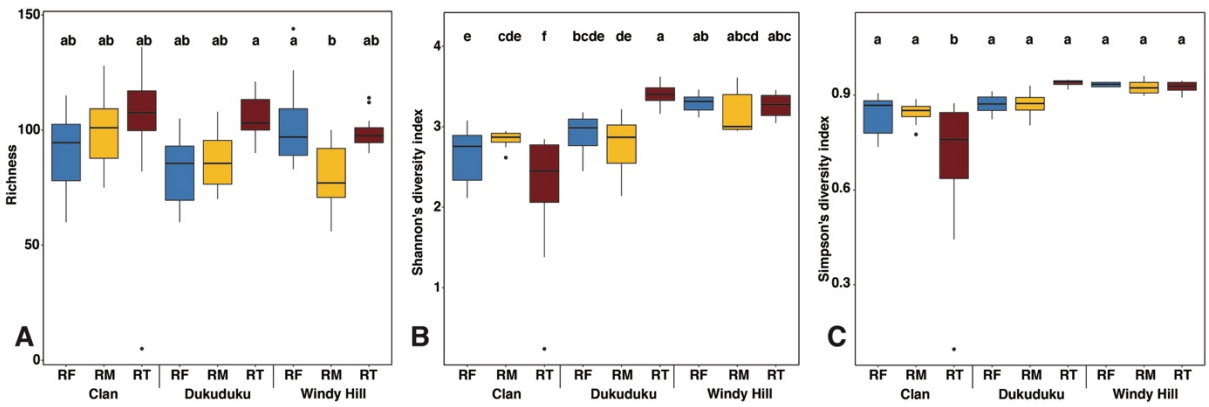

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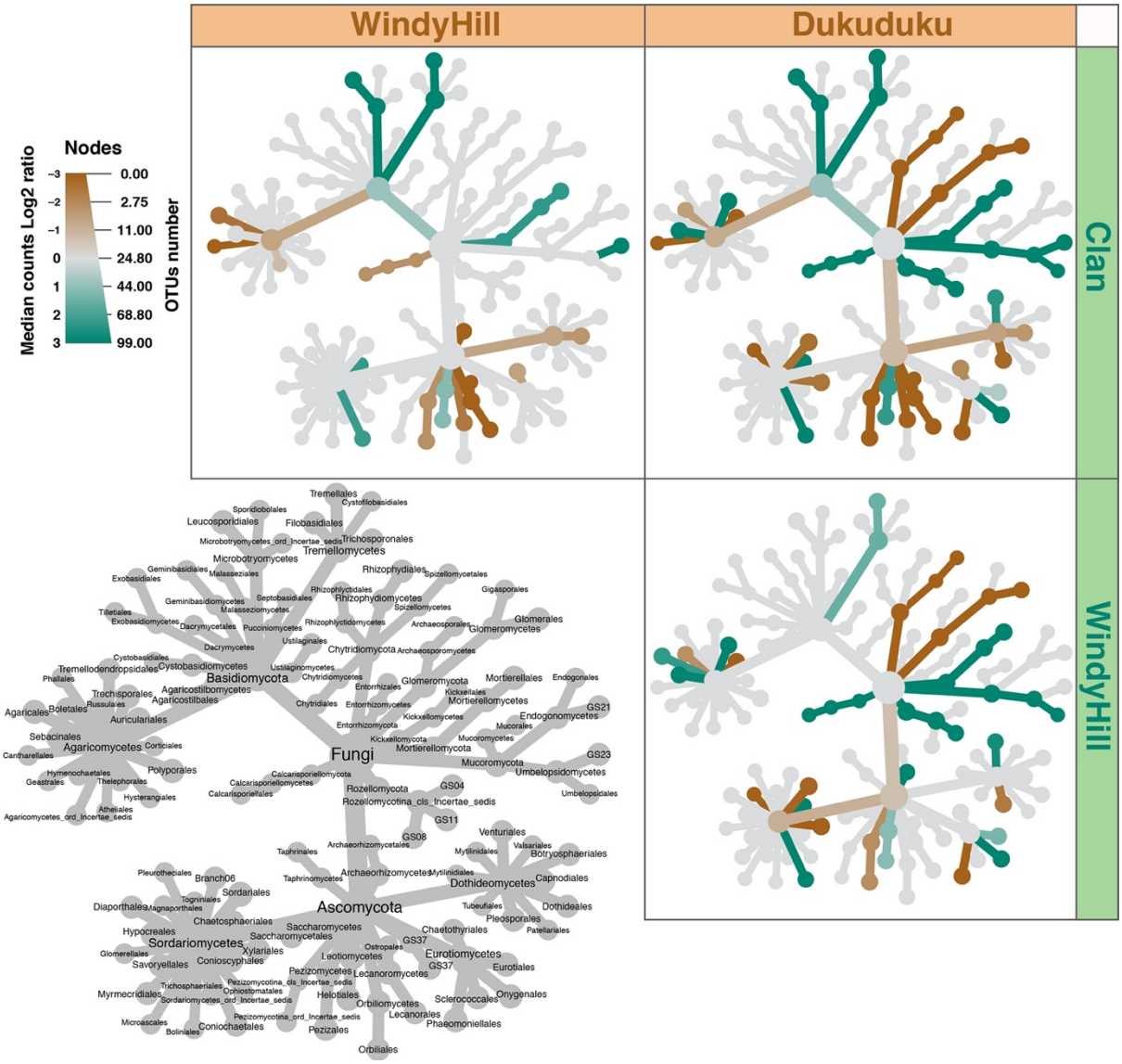

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