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RESEARCH ARTICLE

Land use change rather than surrounding vegetation affects fungal endophyte assemblages in the African wild olive

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Abstract
Context Land use change can significantly affect plant-fungal interactions.
Objectives We assessed how fungal endophytes within African wild olive (*Olea europaea* subsp. *cuspidata*) twigs are influenced by different levels of land use change and differences in surrounding vegetation types.
Methods Twigs were sampled in the Western Cape Province (South Africa) and their fungal endophyte assemblages were characterised using culture-independent DNA metabarcoding. We assessed the effects of land use change (natural, semi-natural and planted (completely transformed)) and differences in surrounding vegetation types (grasses/low-growing plants versus shrubs/trees versus other olives) using fungal endophyte alpha and beta diversity measures. Co-occurrence networks were constructed to assess assemblage connectivity under different scenarios and to identify OTUs of potential ecological significance.
Results OTU richness, but not abundance, was significantly influenced by both land use change and differences in the surrounding vegetation types. Planted African olives and those surrounded by heterospecific trees harboured the highest OTU richness. Only levels of land use change significantly influenced fungal endophyte assemblage composition. Specifically, fungal assemblages from natural habitats were distinct from those in planted and semi-natural habitats, which were similar to each other. Co-occurrence network analyses revealed that cohesive and species rich networks could only be maintained within the natural habitats.

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Conclusion These findings suggest that although the African olive is widespread, the identity and composition of their associated fungal assemblages are particularly sensitive to land use change. This study highlights the importance of conserving natural habitats, not just for the plants, but also for the maintenance of their associated fungal endophytes.

Keywords High-throughput sequencing · Landscape heterogeneity · Core Cape Subregion · Plant-fungal interactions · *Olea africana*

Introduction

Human-mediated disturbances such as urbanisation, agricultural activities and climate change are driving biodiversity loss at an unprecedented rate (Tilman et al. 2001; McKinney 2002; Albrecht et al. 2007; Komatsu et al. 2019). Legacy effects of these disturbances remain in the landscape for many years and continue to impact ecological processes (Foster et al. 2003; Krauss et al. 2007). Land use activities can have cascading consequences on ecosystems including biodiversity loss and breakdown of symbioses (Vanbergen and The Insect Pollinators Initiative 2013; Komatsu et al. 2019; Truchy et al. 2019). Today, negative consequences of anthropogenic change for habitat quality and structure have been documented for virtually all major groups including plants, animals, bacteria and fungi (Weiner et al. 2014; Nguyen et al. 2016; Hyvärinen et al. 2019; Leveau 2019).

Anthropogenic disturbances and their legacy effects are often documented for plant composition and ecosystem functioning (Lloret and Vilà 2003; Komatsu et al. 2019; Abadie et al. 2020). Increasingly, however, human-mediated land use change (LUC) has been shown to have major implications also for other taxa (Fischer and Lindenmayer 2007; Crouzeilles et al. 2016). For example, overall arthropod and microbial diversity usually decrease when exposed to anthropogenic disturbances (Matsumura and Fukuda 2013; Yekwayo et al. 2016, 2017). However, responses of different taxa may vary to the same disturbance making general conclusions in terms of the effect of LUC on biodiversity difficult (Swart et al. 2020).

Efforts to mitigate the effects of LUC on natural ecosystems by restoring and conserving native flora in green belts and gardens in urban environments are advocated. This has proven fruitful for the recruitment of many taxa dependent on native flora such as birds and insects (Forup et al. 2008; Frick et al. 2014; Mnisi 2017). However, there is a sparsity of studies on the benefits of restored areas and green belts to conserve ecosystem processes because ecological processes and biological interactions are slower to recover (Morgan and Short 2002; Ruiz-Jaén and Aide 2005a). Ecosystem processes such as litter turnover and decomposition play a critical role in re-establishing faunal and floral communities and interactions (Ruiz-Jaén and Aide 2005b). The limited available evidence suggests that these altered environments often fail to maintain much of the specialised native organisms, instead favouring generalist taxa (Winfree et al. 2011).

While responses of many taxa to LUC have been well documented, its effects on native plant-associated microbes have received less attention. Yet, plant responses to LUC can depend on their associated fungal assemblages (Franco et al. 2017; Grilli et al. 2017). For example, fungal assemblages may shift to include buffering species that shield or break down pollutants in the landscape, thus allowing the host to persist (Deram et al. 2011; Varela et al. 2015, 2017; Srivastava et al. 2017). Conversely, LUC may be responsible for the breakdown of beneficial symbiotic relationships (Crockatt 2012; Hewitt et al. 2016; Panayotov et al. 2017; Boeraeve et al. 2019). Characterising plant-associated fungal assemblages and their response to LUC is crucial, as fungi can strongly influence ecosystem structure and functioning by serving as decomposers, plant mutualists and pathogens (Orgiazzi et al. 2012; Stone et al. 2018). In addition, they are particularly sensitive to changes in their substrates, to the extent that they have been used as bioindicators of ecosystem resilience/vulnerability to LUC (Jumpponen and Jones 2010; Orgiazzi et al. 2012; Hewitt et al. 2016; Wu et al. 2021). Saproxylic and arbuscular mycorrhizal fungi are especially effective as indicators of general forest health (Siitonen et al. 2005; Abrego and Salcedo 2014; Gáfriková et al. 2020). Plant-associated fungal endophytes (inhabiting internal tissue of plants without causing obvious damage), are also increasingly used as bioindicators in a wide range of ecosystems (Arnold and Lutzoni 2007; Jumpponen and Jones 2010; Kandalepas et al. 2015).

Plants acquire most of their fungal endophytes from the surrounding environment, therefore these microbes can provide valuable information about their hosts' surrounding environment (Saikkonen et al. 1998; Christian et al. 2016; Giauque and Hawkes 2016). In ecosystems function, microbes aid in ecosystem restoration, enhancing resilience of plant communities, and contribute to adaptive strategies (Barea et al. 2002; Singh and Mondal 2018). To this effect, the presence of certain endophytic fungi has been linked with increased tolerance of some plants to stressful environments (Kuldau and Bacon 2008; Deng and Cao 2017). For example, in a manipulation experiment, dark septate endophytes, *Embellisia chlamydospora* and *Cladosporium oxysporum* isolated from the desert shrub, *Hedysarum scoparium,* aided with drought tolerance by influencing root formation (Li et al. 2019). Fungal endophytes can increase the ability of the host to withstand extreme conditions by either producing compounds that aid in stress tolerance or by inducing physiological changes that increase host fitness (Claeys and Inze 2013; Lugtenberg et al. 2016; Molina-Montenegro et al. 2016). However, endophytes may remain neutral until triggered by changes in surrounding environment after which they may become beneficial or even harmful to their host (Saikkonen et al 1998; Slippers and Wingfield 2007). It is therefore important to study fungal endophyte assemblages in both natural and disturbed environments. Understanding changes in fungal endophyte assemblages due to LUC can help us understand how these symbioses contribute to ecosystem resilience.

The aim of this study was to characterise the fungal endophyte assemblages found within twigs of *Olea europaea* subsp. *cuspidata* (African olive) native to the Core Cape Subregion in the Western Cape Province, South Africa using culture-independent high-throughput sequencing (HTS). Fungal endophytes in the twigs of the closely related European olive (*Olea europaea* subsp. *europaea*) are often more diverse (Gomes et al. 2018; Costa et al. 2021) or at least as diverse as in the leaves (Martins et al. 2016). In addition, twigs are hardy, thus presumably less prone to damage from external biotic and abiotic influences and more permanent than leaves making them ideal organs within which to study endophyte assemblages associated with African olive trees. We assessed how differences in LUC affected fungal endophyte alpha and beta diversity found within African olives twigs. Specifically, we compared assemblages from trees growing in undisturbed (natural) areas, those naturally occurring in green belt areas (semi-natural) and those planted in highly transformed habitats such as gardens (Newbound et al. 2010; Tyburska et al. 2013). We hypothesised that olives planted in highly transformed habitats (where exposure to familiar fungal endophytes is least likely out of the three scenarios) will harbour the lowest fungal endophyte richness while those in the natural habitats will harbour the highest richness (Tejesvi et al. 2013; Pickles et al. 2015). We also assessed how differences in vegetation types that surround the focal tree affect endophytic fungal alpha and beta diversity. Specifically, we assessed how endophytic fungal assemblages in twigs of olive trees surrounded by conspecific trees compare to those surrounded by heterospecific trees or when surrounded only by low-growing grassy and shrubby vegetation. We hypothesised that fungal endophyte richness would be highest in trees surrounded by heterospecific trees, since a greater diversity of plants in the surrounding vegetation often correlate to higher microbial diversity (Thoms et al. 2010; Steinauer et al. 2016).

We constructed co-occurrence networks for the factor(s) that significantly influenced fungal beta diversity in the African olive. This was done because complexity in co-occurrence networks has been associated with the resilience of microbial assemblages to environmental perturbation (Rybakova et al. 2017; Santolini and Barabási 2018; Fernández-González et al. 2020). We hypothesised that co-occurrence networks of fungal endophytes from olives planted in highly transformed environments would show the least complexity and have the fewest significant co-occurrences. Conversely, the samples from the natural habitats (where there was no LUC) would show the highest network complexity. Characterising the relationship between fungal endophytes, their hosts and the environment will improve our understanding of the consequences of changes in ecosystems structures in the anthropogenic era and may prove useful for the commercial production of olives in the Core Cape Subregion.

Methods

Host and site selection, and sampling design

Olea europaea subsp. *cuspidata* is one of six subspecies in the *Olea europaea* complex, alongside the widely cultivated *Olea europaea* subsp. *europaea* (Besnard et al. 2007a). These plants, previously used as root grafts for cultivated olives, are important features of the natural landscape and are also used for ornamental and ethnobotanical purposes (Besnard et al. 2007b; Long et al. 2010; Masoko and Makgapeetja 2015; Aumeeruddy-Thomas et al. 2017). The African olive naturally occurs across a wide range of habitats such as ravines, woodlands, forest edges and kloofs (Palgrave 1977; Palmer 1977). In the renosterveld vegetation type of the Fynbos Biome (component of the Core Cape Subregion) the African olive often forms "fynbos thickets" where it natural grows abundantly resulting in conspecific patches (Mucina and Rutherford 2006). This plant is also often planted in residential gardens, parks and roadsides. Due to its resilience, even after transformation of its natural habitats this plant is still often encountered within altered habitats such as alongside river canals and rivers passing through towns (*Pers. obs.*). This makes it an ideal focal plant to study the effects of LUC (natural, semi-natural and planted) and differences in surrounding vegetation type (conspecific trees, heterospecific trees and shrubby/grassy vegetation) on fungal endophyte assemblages.

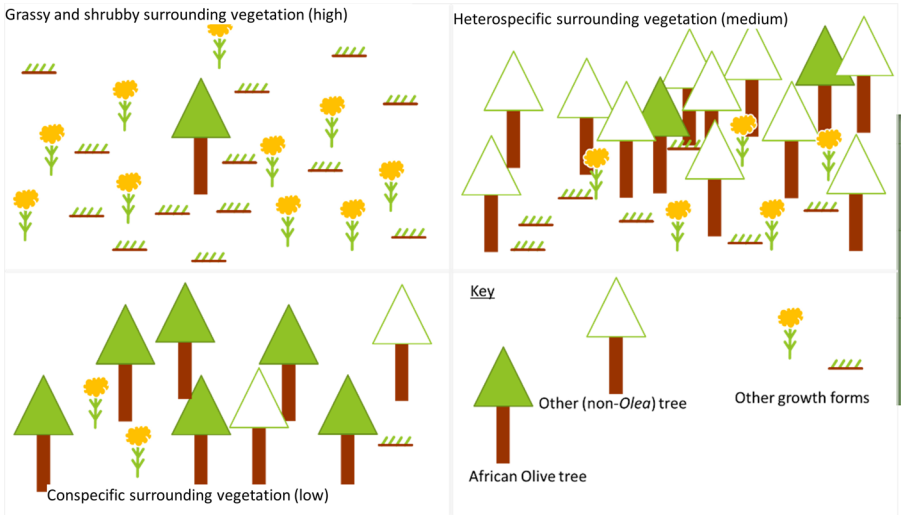
Sampling was conducted in the south-western region of the Western Cape Province, South Africa. This region has a Mediterranean climate with cold and wet winters, and hot and dry summers (Born et al. 2007). The Western Cape is agriculturally important in South Africa (Archer et al. 2019), leading to the transformation of a large portion of the natural systems for agricultural purposes. Like many major metropolitan areas, Cape Town and the Cape Winelands areas have also seen an increase in urbanisation, which has further contributed to the increase of natural ecosystem's transformation. Twigs of the African olive were collected from Stellenbosch (n = 21 trees), Paarl (n = 21 trees) and Somerset West (n = 21 trees). These sites were chosen as they are geographically close to each other with similar soils, climate and anthropogenic LUC types, and they possessed the full spectrum of the LUC and surrounding vegetation categories of interest. Additionally, a previous study indicated that fungal endophyte assemblages found in olive twigs from these locations were not affected by distances between sites, thus removing geographic distance as a possible confounding factor (Ngubane et al. 2023).

Trees were selected based on the levels of experienced LUC in the habitat within which they grew (natural, semi-natural or planted), defined as follows:

- Natural (no LUC)—the selected tree individual grew naturally in undisturbed natural habitats (without any land use activities) and protected area (such as nature reserves).
- Semi-natural (intermediate LUC)—the focal tree grew naturally in an area with high habitat transformation. For example, where olive trees grew along a riverbank in a green area in a town surrounded by roads and other urban infrastructure.
- Planted (most extensive LUC)—the sampled tree was planted in an area with high levels of land use activities such as urban parks, urban roadsides or gardens.

Within each of the LUC categories, tree individuals were chosen based on differences in surrounding vegetation types, with the surrounding environment circumscribed to within ca. 20 m around the tree of interest (Fig. 1). We prioritised consistency across the categories and sampling as far as possible:

- Low difference in contrast to surrounding vegetation—Low growth form contrast with the surrounding vegetation. The focal tree was surrounded by conspecific trees (≥ 10 olive trees within 20 m of the focal tree). Typically canopies of trees were touching or within 5 m from the focal tree.
- Medium difference in contrast to surrounding vegetation—Medium growth form contrast with the surrounding vegetation. The focal tree was surrounded by at least 10 heterospecific trees of a similar height, with no other olives. Typically canopies of trees were touching or within 5 m from the focal tree.
- High difference in contrast to surrounding vegetation—High growth form contrast with the surrounding vegetation. The olive tree was sur-



		NATURAL	SEMI-NATURAL	PLANTED
LOW	P	3	3	2
	STB	1	3	4
	SW	3	1	1
MEDIUM	P	3	3	1
	STB	1	1	3
	SW	3	3	3
HIGH	P	3	2	1
	STB	2	3	3
	SW	2	2	3

Fig. 1 LEFT: Graphic depiction of the criteria used to select the African olive tree to sample in the context of the surrounding vegetation. RIGHT: The number of trees sampled per site within the nine categories of land use change and differences in surrounding vegetation type. Paarl (P) = 21, Stellenbosch (STB) = 21 and Somerset West (SW) = 21

rounded by low-growing shrubby and grassy vegetation, with no other trees in sight.

Sampling followed a full factorial design with nine categories that covered both the LUC and differences in surrounding vegetation types (Fig. 1).

We collected samples from one to four trees (3 to 5 m height and at least 50 cm diameter) per site such that each of the nine categories defined above contained seven samples (Fig. 1). Sample number per locality was dependent on the number of individuals found that conformed to the definitions. Most of the sampling was conducted on 9 April 2018 (45 trees). Later (10 July 2018), a total of 18 trees were added to increase sample size. These samples were spread out evenly across sites and categories. Tree size was standardised as much as possible by collecting samples from those with a diameter of at least 50 cm. Focal individuals were a minimum of 200 m apart and the "surrounding environment" was assessed within an approximate 20 m. From each plant, four twigs (*ca*. 5 mm in diameter and *ca*. 10 cm long), with no visible disease symptoms, were collected from the previous growing season, one from each of the four cardinal directions (N, E, S, W). Samples were frozen at – 80 °C prior to processing. Twigs were surface sterilised in 70% ethanol (45 s), household bleach (60 s), 95% ethanol (30 s) and then rinsed with autoclaved double distilled water for 30 s (Slippers and Wingfield 2007; Moral et al. 2010). Approximately 1 cm length of twig was aseptically excised from the middle of each twig and the four pieces per tree were pooled for DNA extraction.

DNA extraction

Samples were ground into fine powder using a mortar and pestle, cleaned between samples using 70% ethanol, household bleach and autoclaved double distilled water. DNA extraction followed a modified Doyle and Doyle (1990) protocol. Modifications included using 2 µl mercaptoethanol (instead of 1 µl) with 500 µl 2×CTAB buffer (supplemented with 5 µl RNAse A (120 U/mg) and 7 µl Proteinase K (2.5 U/mg)). Extracted DNA was washed twice with 100 µl double distilled water, 75 µl of 5 M KAc and 700 µl of ice cold 70% ethanol.

Library preparation

Library preparation was conducted in two multiplex polymerase chain reactions (PCR). PCR1 and PCR2 were conducted to add internal transcribed spacer (ITS) primers carrying tags and indexes carrying Illumina sequencing adapters, respectively. In the end,

ITS amplicons carried a unique combination of tag and index corresponding to the sample of origin.

During PCR1, ITS1F (5′-CTTGGTCATTTAGAG GAAGTAA-3′, Gardes and Bruns 1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC- 3′, White et al. 1990) primers modified for multiplex barcoding (metabion®, Planegg, Germany) were used to amplify the fungal ITS region from total DNA extracted from olive twigs. PCR volumes (12.5 µl per sample) contained 6.25 µl GoTaq® G2 Hot Start Colorless Master Mix (Promega, Fitchburg, USA), 0.25 µl of 0.1 µM per primer, 5.25 µl ddH_2O and 0.5 µl template DNA. PCR reactions were conducted using a BIO-RAD DNA Engine® thermocycler (BIO-RAD Laboratories Inc., Hercules, USA) under the following conditions: an initial denaturation step at 95 °C for 3 min, followed by 32 cycles (denaturation at 94 °C for 27 s, annealing at 57 °C for 1 min, and elongation at 72 °C for 90 s) and then termination with a final elongation step at 72 °C for 7 min. The ExoSAP protocol (New England BioLabs Inc., Ipswich, USA) was followed to remove excess DNA, and primers and their homo- and heterodimers. During PCR2, Illumina adapters and indices were added onto PCR1 products. PCR2 volumes (25 µl per sample) contained 12.5 µl GoTaq, 0.5 µl of 0.1 µM of each primer, 6.5 µl ddH_2O and 5 µl PCR1 product. PCR reaction conditions were identical to those used for PCR1, except that only 5 cycles were conducted instead of 32 cycles.

The PCR products were sequentially pooled until amplicons of all 63 samples were in a single tube. Band intensities (used as proxy of molarity) were quantified using ImageJ version 1.52a (Ferreira and Rasband 2012). Equimolar pools (with similar intensities) were combined. The resulting pools were purified using the CleanPCR® Kit (CleanNA, Waddinxveen, Netherlands). This final pool was sent for sequencing at the Genetics Department, Ludwig Maximilian University, Munich, using the MiSeq Reagent Kit v3 for 2×300 Illumina MiSeq® sequencer (Illumina Inc., San Diego, USA).

Sequence cleaning, identification, and quantification

The batch of sequences was subjected to quality control and demultiplexing using the QIIME 1.9.1 pipeline (Caporaso et al. 2010). Only forward reads were used for subsequent analyses. Sequences were separated and assigned to their corresponding sample of origin based on their tag-index combinations and the reference mapping file. Once separated, the tag-index sequences were trimmed using the FASTX-Toolkit (v. 0.0.13, http://hannonlab.cshl.edu/fastx_toolkit/). The ITS sequences were screened for possible chimeras using an abundance-based method in the USEARCH platform (Edgar 2010). The remaining ITS sequences were grouped into operational taxonomic units (OTU) based on sequence similarities (97% similarity threshold) using CD-HIT-OTU (http://weizhongli-lab.org/cd-hit-otu/; Stackebrandt and Goebel 1994; Li et al. 2012). Representative sequences were used for taxonomic placement using QIIME and the UNITE v. 7.2 database (Kõljalg, et al. 2013). The OTUs that had no BLAST hits based on the UNITE database were queried using the basic local alignment tool (BLAST) located in GenBank within NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Fungi that could not be placed using the UNITE database, a 97% sequence similarity cut-off was applied to identify the closest matches using BLAST. If an OTU had previously only been detected using culture-independent methods and has never been placed at any taxonomic level during these explorations, then they are labelled "uncultured fungus."

An OTU table was constructed with the number of reads used as a proxy for fungal abundance. However, it is worth noting that using the number of reads to infer abundance can be biased as some ITS primers have been found to amplify some fungal taxa with more ease compared to others (Bellemain et al. 2010; Tedersoo et al. 2015). Additionally, during sequencing, sequencing depth can vary significantly even within the same sequencing run (McMurdie and Holmes 2014). Thus, in the present study, we also considered relative abundance and rarefied richness during our analyses.

Analyses of fungal endophyte diversity within the African olive

Fungal endophyte alpha diversity response to LUC and differences in surrounding vegetation

Fungal diversity within the African olive was calculated using the non-parametric Chao2 and Jackknife2 species estimators (Chao et al. 1992; Hortal et al. 2006) using Primer6 (Anderson et al. 2008). Total and core fungal endophyte richness, rarefied richness,

abundance and relative abundance were compared between the LUC categories and categories of surrounding vegetation using modelling procedures in R v. 3.1.2 (R Development Core Team 2015). Core fungal taxa were defined as those OTUs that appeared in at least 50% of the samples. The roles of LUC and differences in surrounding vegetation in OTU richness and core richness were assessed using generalised linear modelling with a Laplace approximation fitted with a Poisson family distribution using the *lme4* package in R (Bates and Sarkar 2007). This model was selected as the data were not normally distributed based on the Shapiro-Wilks test in *nortest* and histogram plots (Gross and Ligges 2015). The model was run with and without site as the random effect, and then the model with the lowest Akaike Information Criterion (AIC) was selected. The random effect, site, was used to account for overdispersion and possible spatial autocorrelation. Models contained effects of LUC and surrounding vegetation. Rarefied richness, on the other hand, was normally distributed, and showed no signs of overdispersion or spatial autocorrelations. A linear model was thus used to compare rarefied species richness between the categories and their interactions (without site as a random variable). Models were used to compare abundances and core abundances of different categories and their interactions. Where main tests were significant, conservative Tukey post hoc tests were conducted using the *multcomp* package (Hothorn et al. 2008).

Assessment of fungal endophyte assemblage composition and turnover within and between LUC categories and different vegetation types in the surrounding

Beta diversity was analysed using Primer6 (Anderson et al. 2008). Beta diversity between different LUC categories or categories of surrounding vegetation type (β1) and within these levels (β2) were assessed using permutational multivariate analyses of variance (PERMANOVA) and permutational multivariate analyses of dispersion (PERMDISP), respectively. PERMANOVA analyses were conducted considering both the fungal abundances and richness while PERMDISP analyses focused on fungal richness only. The abundance-based dataset was square-root transformed and a Bray–Curtis dissimilarity matrix was generated before performing a PERMANOVA with 999 permutations (Anderson 2001). The PERMANOVA analyses based on the presence and absence dataset, were performed with 999 permutations on a Jaccard's dissimilarity transformed matrix (Magurran 2004). Post hoc comparisons for significant effects were performed using the pairwise PERMANOVA test. PERMDISP analyses were performed on a Jaccard dissimilarity matrix using 999 permutations (Anderson 2001). These β1 and β2 diversity analyses were also performed to assess the effect of LUC and differences in surrounding vegetation type on the core fungal assemblages. For all significant main effects, pair-wise comparisons and canonical analyses of principal coordinates (CAP) were conducted. CAP analyses were conducted using the R package, *Phyloseq* v.1.28.0 (McMurdie and Holmes 2013) in R.

We constructed co-occurrence networks for significant predictors. Specifically, we identified taxa likely to be important to fungal assemblages of the African olive. Co-occurrence analyses were only conducted for the factor(s) with significant influences to shaping fungal endophyte assemblages of the African olive. Significant co-occurrences were calculated based on Spearman's correlation coefficients (significant when Pearson's $\rho > 0.5$ (Spearman 1904) and p-value < 0.05) with a Benjamini–Hochberg standard false discovery rate (FDR) correction for multiple comparison (Benjamini and Hochberg 1995) using *Hmisc* in R (Harrell and Dupont 2007). Fungal co-occurrences that appeared more times than they would by chance were visualised in a co-occurrence network using Cytoscape v3.7.2 (Cline et al. 2007). UNITE OTU classifications were used to label nodes. To summarise similarities and differences between and within categories considering all taxa, similarity percentages (SIMPER) analyses were conducted in Primer6 and reported as an accompanying summary to the networks.

A collection of network descriptive measures (such as average node degree, total number of nodes, total number of edges, shortest path length, network diameter, clustering coefficient, graph density and modularity) were calculated and reported to describe the networks presented (Assenov et al. 2008; Newman 2003, 2006, 2010). The number of nodes or vertices (v) signifies the number of species (or OTUs) in a network, and these nodes are connected by edges. Node Degree indicates the number of other nodes each node is directly connected to in the network (Assenov et al. 2008; Newman

2010). The shortest distance (fewest number of edges) it takes to achieve the connections (direct and indirect) between all nodes in each network is called the short path length (spl) (Newman 2010). On the other hand, network diameter (nd) is measured as the longest path connecting any pair of nodes that are directly or indirectly connected (Newman 2010). Some nodes may be more connected to each other than they are to the rest of the network, and thus create a subnetwork within the bigger network. This tendency is referred to as modularity (Newman 2003, 2006). The clustering coefficient (cc) provides an indication of cliquishness of nodes in a network, as it measures the likelihood of connected nodes being part of a subnetwork connected to the large network (Watts and Strogatz 1998). In a network some nodes will be more influential than others, this is referred to as centrality (Delmas et al. 2019). The proximity of a node to all the other nodes in a network is called closeness centrality (C_C; Freeman 1978). Thus, the more central (higher C_C) a node is, the closer it is to all other nodes. Some nodes play a large part in connecting different components of the network, this is called betweenness centrality (C_B; Freeman 1977). Betweenness centrality reflects which nodes are strategically placed and serve as bridges/mediators through which many paths pass to connect the node clusters on either side of the mediator.

Nodes/species that play a bigger role, than most nodes in the network, to keep the network connected and as dense as it appears are referred to as hubs (Delmas et al. 2019). When the closeness centrality of a node is higher than the average closeness centrality it is likely that it is a hub (van der Heijden and Hartmann 2016; Delmas et al. 2019). When a node has a higher node degree than the average nodes (average node degree, ad) in the network, this node also has the potential to be a hub (Agler et al. 2016). Nodes that qualify on more than one of these properties are more likely to be hub species that have the potential to serve an important biological function to either the host, the mycobiome or both.

Results

Fungal endophyte alpha diversity response to levels of LUC and differences in surrounding vegetation types

A total of 491 988 sequences were obtained from 63 olive samples. The sequences belonged to 311 fungal OTUs (Tables S1 and S2). Species richness was significantly influenced by levels of LUC in the habitat (Fig. 2A, Tables 1 and S3), although none of the post hoc comparisons were significantly different (Fig. 2A, Table S3). Differences in surrounding vegetation types played a critical role in species richness within olive twigs (Table 1). Olives surrounded by conspecific trees had significantly lower species richness than those surrounded by heterospecific trees or grassy/ shrubby vegetation (Fig. 2B, Tables 1 and S4). Twigs from trees growing amongst heterospecific trees had the highest fungal richness (Fig. 2B, Table S3). The interaction between LUC and differences in surrounding vegetation types also significantly affected species richness within olive twigs (Table S4). Rarefied richness was not significantly influenced by either LUC or differences in the surrounding vegetation. Core fungal richness was only significantly affected by LUC (Table 1), but none of the pairwise comparisons were significant (Table S3, Fig. 2C). In contrast, fungal abundances (total, relative and core fungal abundance) were neither significantly influenced by LUC nor by differences in surrounding vegetation types (Table 1).

Fungal endophyte assemblage composition and turnover within and between LUC categories and differences in surrounding vegetation types

LUC was an important factor when explaining differences in core and full fungal assemblages, using both Jaccard- and Bray–Curtis dissimilarities (β1, Table 2). The significant effect of LUC (β1 based on Jaccard dissimilarity) on the full and core fungal assemblages was driven by the significant differences between assemblages from olive trees planted in gardens and in natural habitats (Table S5). Post hoc β1 (Bray–Curtis dissimilarity) revealed that whole fungal assemblages were also significantly different between the planted and naturally occurring trees (Table S5). The core fungal assemblages (when considering Bray–Curtis dissimilarity) from the natural habitats were distinct from the assemblages from the planted trees and those from the semi-natural habitats (Table S5). Ordination analyses reflected these groupings and patterns (Fig. 3). Surrounding vegetation type did not influence the core and full fungal assemblage composition (based on Bray–Curtis and Jaccard dissimilarity) within olive twigs (β1, Table 2).

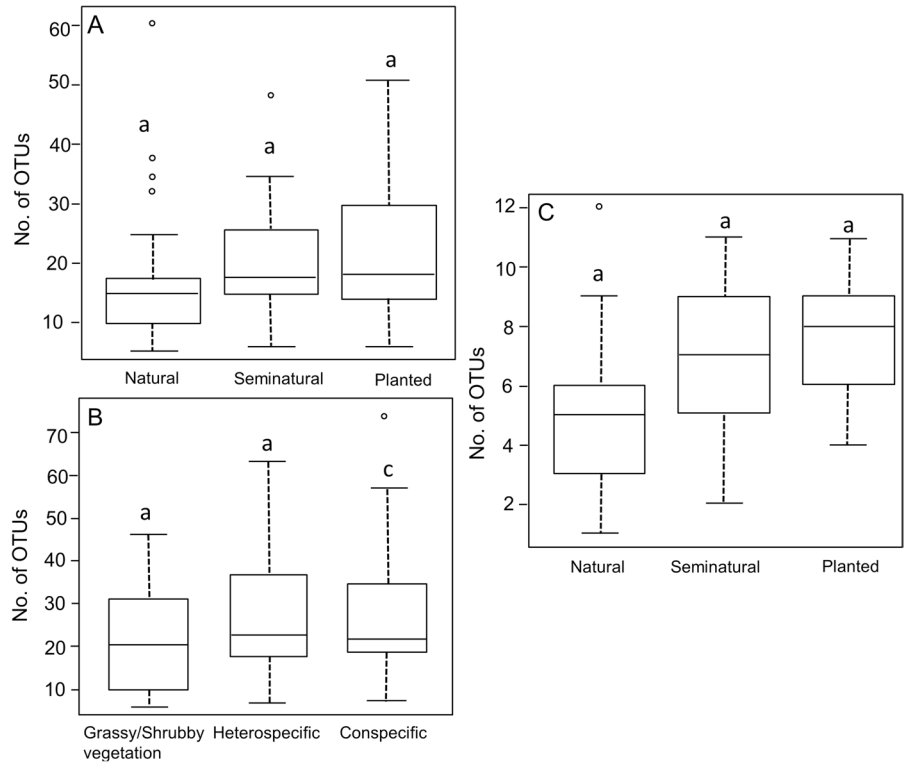


Fig. 2 Box and Whisker plots of species richness in the African olive growing in habitats with different levels of land use change (**A**); African olive surrounded by different vegetation types (**B**) and the core OTU richness according to LUC categories (**C**). Post hoc comparisons were significant at $p < 0.05$ and are denoted by differences in lower-case letters

Table 1 Summary results of linear models of the effect of land use change (LUC) and differences in surrounding vegetation types on the total and core fungal endophyte richness and abundance

	Richness			Rarefied richness			Richness (Core)		
Factor	Wald's Chisq	Chi Df	p	F	Df	p	Wald's Chisq	Chi Df	p
LUC	59.777	6	< 0.001*	0.528	2	0.593	13.339	6	0.040*
Vegetation type	65.498	6	< 0.001*	0.006	2	0.994	6.630	6	0.357
Interaction	49.322	5	< 0.001*	0.350	4	0.843	4.717	5	0.339
	Abundance			Relative abundance			Abundance (Core)		
Factor	LR Stat	df	p	F	Df	p	Wald's Chisq	Chi df	p
LUC	5.769	6	0.450	0.243	2	0.786	2.064	6	0.611
Vegetation type	4.486	6	0.611	2.655	2	0.079	2.028	6	0.450
Interaction	3.283	5	0.511	0.319	4	0.864	2.198	5	1.000

Where the main effects were significant, post hoc test results are presented in Table S3

Tests considered significant if $p < 0.05$ (*)

* indecates the significant results

Dispersion within groups (β2) was only significantly different between habitats with different LUC categories when considering the full complement of taxa but not core taxa (Table 2). This significance was facilitated by the within groups variation in fungal assemblages from the natural habitat, which had a significantly higher average within-group dispersion than the fungal assemblages from the planted and semi-natural habitats (Table S5). Species turnover (in core and overall assemblages) was also not

Table 2 Beta diversity results based on PERMANOVA (Jaccard and Bray–Curtis dissimilarity) and PERMDISP (Jaccard dissimilarity) analyses of fungal endophyte assemblages within twigs of the African olive in response to land use change (LUC) and differences in surrounding vegetation, and the interaction between these two factors

PERMANOVA (β1)

	Jaccard			Jaccard (Core)			Bray-Curtis			Bray-Curtis (Core)		
Groups	**df**	**Pseudo-F**	**p**	**df**	**Pseudo-F**	**p**	**df**	**Pseudo-F**	**p**	**df**	**Pseudo-F**	**p**
LUC	2	1.305	0.021*	2	2.332	0.004*	2	1.363	0.031*	2	1.919	0.016*
Vegetation type	2	0.958	0.628	2	0.899	0.549	2	0.850	0.771	2	0.811	0.709
Interaction	4	0.935	0.751	4	0.908	0.628	4	0.828	0.928	4	0.848	0.735

PERMDISP (β2)

	Jaccard				Jaccard (Core)			
	F	**df1**	**df2**	**p**	**F**	**df1**	**df2**	**p**
LUC	3.515	2	60	0.047*	2.750	2	60	0.115
Vegetation type	1.267	2	60	0.3	0.932	2	60	0.475
Interaction	0.796	8	54	0.713	0.673	8	54	0.836

Results considered significant if $p < 0.05$ (*)

Significant post hoc results are reported in Table S4

* indecates the significant results

significantly influenced by differences in surrounding vegetation types.

Similarity percentages (SIMPER) analyses revealed that fungal endophytes from the natural and planted habitats were the most dissimilar while those from planted and semi-natural habitats were the most similar (Fig. 4 insert). SIMPER also indicated that there was a high fungal endophyte turnover between samples within categories. For example, on average, samples from natural habitats only had 14.750% similarity, while those from semi-natural habitats had 22.590% similarity. This level of heterogeneity within the natural habitats together with the dispersion (β2) average of 61.832 (±0.691) suggest that this habitat harbours highly diverse assemblages even between samples (Fig. 4 insert, Table S5).

Since only LUC significantly influenced fungal assemblages of the African olive, co-occurrence networks were constructed for this factor only. Three fungal co-occurrence networks are presented that visualise significant fungal co-occurrences within the three LUC categories: planted, semi-natural and natural. Olive twigs from the natural, semi-natural and planted LUC categories contained 36, 25 and 16 OTUs, respectively, that co-occurred with each other a significant number of times (Fig. 4; Table S6). The co-occurrence network of OTUs from the natural habitats was the most complex followed by that from the semi-natural habitats. Networks of fungal endophytes from the semi-natural and planted habitats were disconnected, with the highest node degrees of three and one at most, respectively. In contrast, the network of assemblages from the natural habitats had the highest average node degree (ad = 6.611) while that of assemblages from the planted olives had the lowest (ad = 1.125, Table S6).

The co-occurrence network from the natural habitats had the highest number of OTUs with properties that make them possible hub species. Specifically, 14 of the 36 nodes from the natural habitats had more neighbours than the average node, indicating taxa with potential to be important hub species within olive trees in this category. Of the top seven key taxa, three could not be placed at genus level and the rest were assigned to *Aspergillus proliferans*, *Ulocladium chartarum*, *Peniophora* sp. and *Paracladophialophora* sp. The highest graph density value reflects the higher level of connectivity in this network compared to the others (Table S6). In the network from the natural habitats, perhaps the most noteworthy taxon was that assigned to *Alternaria eureka*, which was highly connected (nd = 9, higher than ad), had a high betweenness centrality ($C_B = 0.6$) and clustering coefficient ($C_C = 0.6$) suggestive of a hub species that is highly connected and strategically placed to connect different sub-clusters

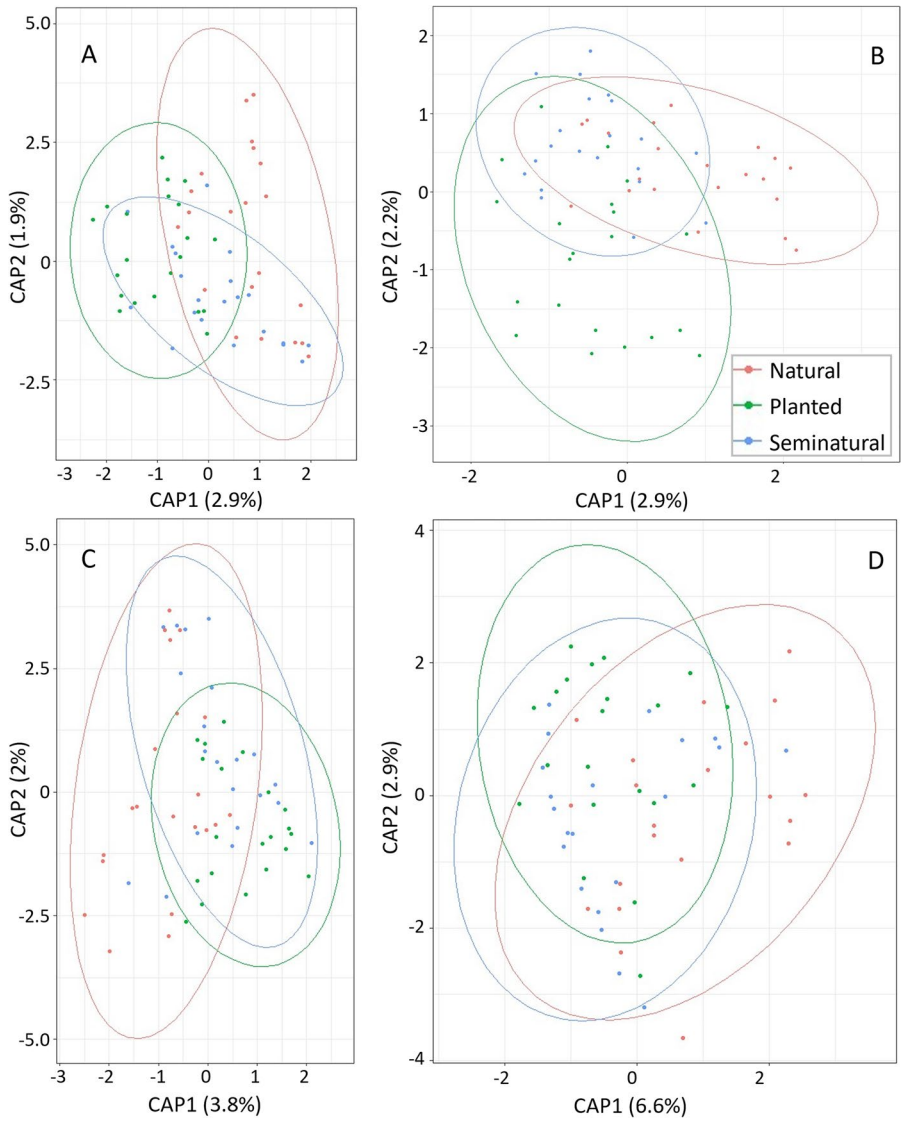


Fig. 3 Canonical analysis ordination (CAP) based on Bray–Curtis (abundance data, **A** and **C**) and Jaccard (incident data, **B** and **D**) dissimilarity of the full (**A** and **B**) and core (**C** and **D**) fungal endophyte assemblages according to land use change categories (planted, semi-natural and natural)

of the overall network. One of the top three highly connected taxa had no BLAST hits on either database, suggesting that this taxon may be undescribed or yet to be captured on either database.

Discussion

This study revealed that land use change has a significant impact on endophytic fungal assemblages within twigs of the African olive (*Olea europaea* subsp. *cuspidata*). However, fungal endophyte richness (alpha diversity) was only significantly influenced by differences in surrounding vegetation types and not LUC (as indicated by post hoc analyses) with trees growing amongst heterospecific trees harbouring the highest richness. The surrounding vegetation had no influence on fungal endophyte assemblage composition (beta diversity) while LUC did. Particularly, fungal endophyte assemblages from the planted olives were distinct from those in the natural habitat. The importance of natural habitats for consistent plant-endophyte associations was highlighted by co-occurrence network analyses where a dense fungal co-occurrence network was only attainable in the natural habitats and completely disintegrated in the most transformed settings (i.e. olives planted in urban areas).

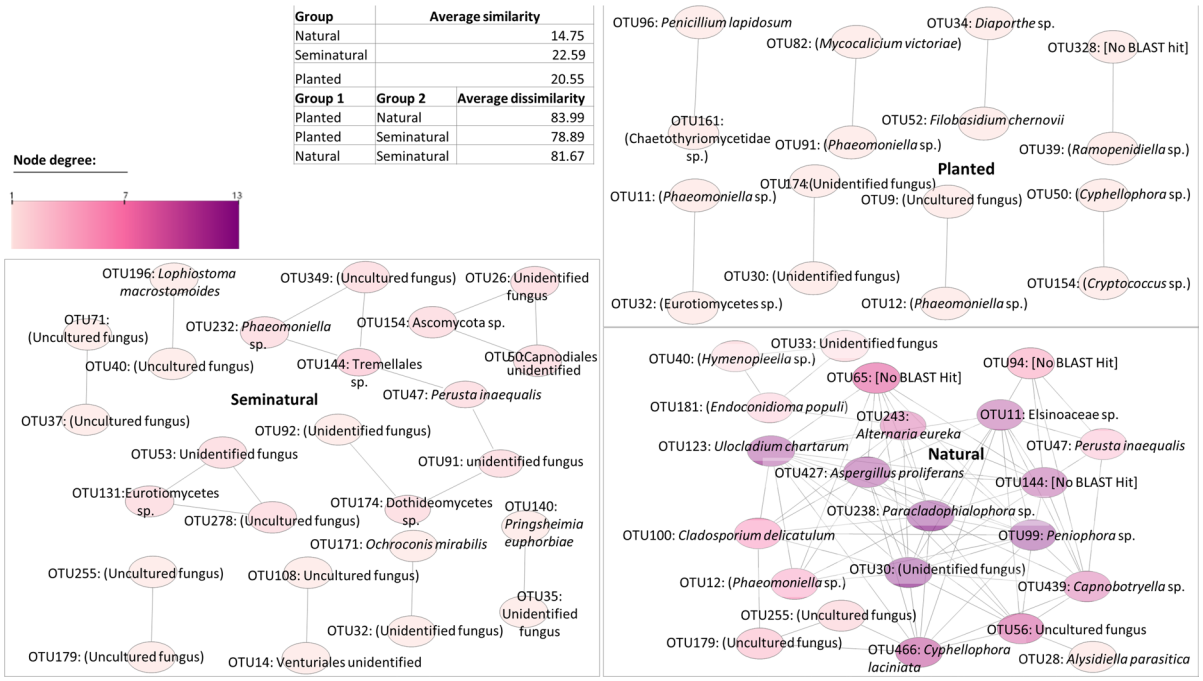


Fig. 4 Co-occurrence networks highlighting significant co-occurrences of fungal endophyte taxa within twigs from planted, semi-natural and natural olive trees. Average similarity and dissimilarity percentages within and between categories are summarised on the inserted table. Species identifications without brackets indicate taxa that were placed using the UNITE database, those in round brackets represent taxa that could not be placed using the UNITE database, but which could be identified using BLAST, while those in square brackets indicate taxa that could not be placed using either database

LUCs often have a negative effect on fungal richness (Deram et al. 2011; Abrego and Salcedo 2014; Boeraeve et al. 2019, 2021). It was therefore surprising that endophyte numbers were consistent in our study across these vastly different impact levels. Our results are echoed by a study where fungal endophyte alpha diversity was similar between *Spartina alterniflora* plants exposed to the Deepwater Horizon oil spill and those that were not (Lumibao et al. 2018). There may therefore be numerous conditions in which some plants can host a similar number of taxa in almost any environment (disturbed or natural). It may be a common phenomenon for plants that are naturally widely distributed, as has been observed in the lodgepole pine, to have a strong host filtering ability (Pickles et al. 2015) such that they keep endophyte richness fairly constant across different LUC levels. In contrast, the surrounding vegetation type had a significant influence on fungal endophyte species richness within the African olive. This was especially evident when focal trees were surrounded by heterospecific trees which likely increases the richness of spores available in a particular habitat and that may already have some adaptation towards infecting trees as opposed to grasses and shrubs (Steinauer et al. 2016; Chen et al. 2020; Redondo et al. 2020). Nonetheless, the fungal endophyte richness in these olives is likely to be much higher since the ITS region used in metabarcoding is known to be conservative (Abdelfattah et al. 2015).

Fungal endophyte assemblage composition (beta diversity) of the twigs of the African olive in natural habitats differed from those in the habitats experiencing increased LUC pressures (semi-natural and planted). This was true irrespective of whether the whole assemblages or just the core assemblages were considered. This indicates that even slight transformation levels (such as was the case for trees growing in green belt areas) could have significant impacts on endophyte assemblage composition. The drastic differences in fungal assemblage composition when habitats experience LUC have also been reported in fungi in forests (Purahong et al. 2014). In the present study, endophyte assemblage composition in the relatively

natural (semi-natural) settings resembled those of trees planted in extremely transformed urban gardens. The changes in assemblage composition between the plants in natural compared to those in transformed areas may be important for the ability of host plants to deal with consequences of anthropogenic activities (Perreault and Laforest-Lapointe 2022), as was the case for fungal endophyte assemblages within roots of *Arrhenatherum elatius* growing in soils contaminated by heavy metals where colonisation intensity by dark septate fungi was noticeably higher in contaminated sites (Deram et al. 2011). *Arrhenatherum elatius* assemblages adapted to changes in LUC by shifting to include taxa with the ability to aid in heavy metal contamination tolerance (Deram et al. 2011). Some of the fungal endophytes in the mycobiome may reflect opportunistic taxa, a proportion of which are able to tolerate the LUC stresses including those that confer a fitness advantage for the host in the disturbed areas with different moisture regimes and pollutants influxes. In addition, the high heterogeneity of the fungal endophyte assemblages, particularly in the undisturbed habitats (compared to olive in semi-natural and planted LUC levels), may be instrumental to the highly adaptive nature and vast geographic distribution of this olive plant.

Our results suggest that urbanisation changes microbial assemblage associations leading to different co-occurrence patterns between natural and LUC conditions. The co-occurrence network of the fungal endophyte taxa from the natural habitat were highly connected, indicative of a small world network (Watts and Strogatz 1998; Newman 2010). Assemblages that form highly connected networks have been found to have higher resilience to changes in their habitat than those with less dense networks (Rybakova et al. 2017; Santolini and Barabási 2018). In the context of the present study, the network from the natural habitat comprised of nodes that were densely connected possessing the smallest shortest path length while the networks from the planted and semi-natural were disconnected without an uninterrupted path to connect all or most of the nodes. Reasons behind the breakdown of plant-fungal associations are many but legacies of human-mediated habitat transformation have been implicated (van Geel et al. 2018; Boeraeve et al. 2019). The high assemblage turnover from one sample to the next within the natural habitat, yet the high connectivity of the network, suggests that the fungal co-occurrences in the natural habitat may serve an important purpose to this host. Highly connected nodes (potential microbial hubs) have been shown to play an important role in plant health because they can mediate the relationships between the members of the microbiome and between the microbiome and the host (Agler et al. 2016). The seven nodes with the highest node degree played an important role in the observed network density, possessing high betweenness centrality. It is interesting that, the disintegrated semi-natural and planted habitat fungal co-occurrence networks were coupled with either the demotion or absence of these seven taxa.

Habitat alteration can disrupt microbial co-occurrence networks (Gao et al. 2022) as it appears to be the case in the present study. Interestingly, Wu et al. (2021) found that co-occurrence networks of fungi formed more connections in the face of perturbation. However, Wu et al. (2021) investigated total fungi in soils while we specifically focused on fungal endophytes taken up by the host. In the context of Wu et al. (2021) soil fungi showed an ability to adjust to changes in the environment by forming stronger attachments while fungal endophytes in the olive appear to struggle in the face of LUC. As indicated by the network analyses, the endophytes do not form cohesive fungal endophyte assemblages in these altered ecosystems. This disconnection has unknown consequences for the olive host. In the disconnected "networks" from the semi-natural and planted habitats, network properties such as hub species, betweenness centrality and closeness centrality held no practical meaning since these "networks" consisted of many disconnected co-occurrences. Even so, many of the taxa that appeared in these disconnected elements co-occurred a significant number of times to suggest that their association with the olive host may serve important functions to the olives in these habitats.

Some fungal endophyte taxa known to associate with plants in areas with land use activities were also represented in the co-occurrence network of the planted African olive. Due to the short length of metabarcoding ITS amplicons and the conservative nature of this marker, it has been advised that fungal placement be limited to genus level (Abdelfattah et al. 2015; Knight et al. 2018). To this effect, we detected three *Phaemoniella* taxa which were abundant and significantly co-occurred with other taxa within the twigs from the planted African olives, while only one

of these was significantly associated with other taxa in olives in the natural habitats. Taxa in this genus are often encountered within agricultural crops, including *O. europaea* subsp. *europaea* (Carlucci et al. 2013; Moral et al. 2017; Gomes et al. 2018) and are implicate as causal agents of Petri disease and esca in grapevine in South Africa (Retief et al. 2006). In addition, *Alternaria* taxa were encountered within the African olive twigs from planted trees and within those cultivated (*O. europaea* subsp. *europaea*) in the country (Ngubane et al. 2023). *Alternaria* has a global distribution and is often encountered in agricultural and forestry crops (Malacrinò et al. 2017; Basson et al. 2019). Some *Alternaria* species have also been associated with stress tolerance in plants, including soil pollution and drought (Rodriguez and Redman 2008). Thus, these taxa may be acquired from the surrounding plants under anthropogenically altered conditions. However, they may not associate with the olive host in natural conditions where this host, and its surrounding vegetation, are not experiencing the same anthropogenic activities as those in the semi-natural and planted habitats. Given that taxa in these genera have a well-known role in adaptation (in the case of positive associations), they may be important to plants in the Core Cape Subregion as we continue to experience consequences of expanding agricultural and urbanisation activities.

It is worth cautioning that co-occurrence alone does not translate to interaction (Blanchet et al. 2020; Goberna and Verdú 2022). Although co-occurrence is important to interaction (Delmas et al. 2019), additional experimental and observational evidence is needed to demonstrate interaction (Araujo et al. 2011; Gao et al. 2022). Nonetheless, species that co-occur more times than they would by chance have a higher likelihood of interacting than those that do not (Delmas et al. 2019). Co-occurrence networks can identify potential keystone species in order to illuminate assemblage responses to anthropogenic changes. In the present study, the taxa identified as potential hub species need further investigations to describe and improve our understanding of their ecological role in the mycobiome of the African olive. These taxa may hold the key to improving the ability of native hosts to thrive in altered habitats. Although restoration efforts have shown success at plant level, microbes often struggle to re-establish (Gooden et al. 2020). Thus understanding the role of different elements of microbial assemblages can inform strategic inoculations of microbes during restoration activities and disease management in the agriculture of the European olive in the Core Cape Subregion.

Conclusion

Fungal endophytes are important to plant health. Yet, these organisms remain amongst the largely neglected groups in studies focused on the effect of LUC on biodiversity, especially in South Africa. Given the different roles they play in ecosystems, including those under anthropogenic influences, they provide a promising tool to add to our arsenal as we attempt to mitigate the consequences of LUC on biodiversity. Their sensitivity to habitat degradation makes studying fungal endophytes, uncovering their diversity in a biodiversity hotspot, and their role in altered habitats a priority. Remnant natural habitats of the African olive appear to be important refugia for microbial taxa (likely native) that may be important for ecosystems' function and integrity. Given the extensive agricultural and urbanisation activities in the Cape Winelands region, the remaining natural pockets may be amongst the few fungal endophyte reserves harbouring high native species richness. Importantly, given the conservative nature of the ITS region (Abdelfattah et al. 2015; Knight et al. 2018) we used to characterise assemblages here, our uncovered species richness is likely an underestimation of the true fungal richness within the African olive in the Core Cape Subregion. Future culture-based studies are needed to confirm the identification of taxa revealed as potential hub species and to describe the new species. An understanding of how existing communities and ecosystems respond to environmental and land use change will help inform landscape management decisions. For example, the α- and β- diversity results presented in this study suggest that although habitat transformation may not affect species richness, it may affect the relative representation of taxa.

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Declarations

Competing interests All authors of this manuscript have no conflict of interests.

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