Research paper

Investigation of the Soil Microbiome of Healthy and Wilted Acacia Trees in a Plantation in Taiwan

Tse-Yen Liu,[1] Hsin-Hui Shih,[2] Jia-Bin Tsai,[3] Chao-Han Chen,[1] Chia-Chen Wu[4,5]

【Summary】

The phytopathogenic fungus, *Fusarium oxysporum* f. sp. *koae*, is recognized as the cause of wilt of *Acacia confusa* seedlings and trees in Taiwan. This study investigated the soil microbiome in an acacia plantation to assess its influence on the health of *A. confusa*. The health of plants is thought to be strongly correlated with soil microbial communities, and *F. oxysporum* is an important soil-borne pathogen. This study analyzed the microbial composition of the rhizosphere soil of both healthy and wilted acacia trees. Rhizosphere soils of healthy and wilted *A. confusa* in the acacia plantation were collected, and then DNA was extracted and sequenced using next-generation sequencing (NGS) to acquire sequences of the fungal internal transcribed spacer (ITS) and the bacterial 16S ribosomal DNA (16S rDNA). Results revealed no significant differences in the fungal or bacterial compositions at the class and genus levels between the soil microbiomes of healthy and wilted trees. Notably, *Fusarium* spp. was not a dominant fungal genus in either sample. Despite the higher microbial diversity observed in healthy trees compared to wilted ones, the difference was not statistically significant. A linear discriminant analysis (LDA) revealed several dominant microorganisms in the soil of wilted trees. These included the fungal genus *Cephalotrichum*, the bacterial phyla Gammaproteobacteria and Proteobacteria, the bacterial order Rhodanobacteraceae, and the bacterial families Kapabacteriales, Diplorickettsiales, and Xanthomonadales. Additionally, the bacterial genera, *Lacunisphaera* and *Chujaibacter*, were found to be prominent in the soil samples of wilted trees. This study represents the first analysis of the soil microbiome in an acacia plantation, shedding light on potential biological factors related to *A. confusa* wilt which may guide future research on microbiome-plant interactions.

Key words: *Fusarium oxysporum* f. sp. *koae*, *Acacia confusa*, vascular wilt, soil microbiome.

Liu TY, Shih HH, Tsai JB, Chen CH, Wu CC. 2023. Investigation of the soil microbiome of healthy and wilted acacia trees in a plantation in Taiwan. Taiwan J For Sci 38(3):221-32.

[1] Forest Protection Division, Taiwan Forestry Research Institute, No. 53 Nanhai Rd., Zhongzheng District, Taipei 100051, Taiwan. 林業試驗所森林保護組，100051臺北市中正區南海路53號。

[2] Lienhuachih Research Center, Taiwan Forestry Research Institute, No. 43 Hualong Lane, Yuchih Township, Nantou 555002, Taiwan. 林業試驗所蓮華池研究中心，555002 南投縣魚池鄉五城村華龍巷43號。

[3] Chiayi Research Center, Taiwan Forestry Research Institute, No. 65, Lane 432, Wenhua Rd., Chiayi 600054, Taiwan. 林業試驗所嘉義研究中心，600054 嘉義市西區文化路432巷65號。

[4] Silviculture Division, Taiwan Forestry Research Institute, No. 53 Nanhai Rd., Zhongzheng District, Taipei 100051, Taiwan. 林業試驗所育林組，100051臺北市中正區南海路53號。

[5] Corresponding author, E-mail: chiachen@tfri.gov.tw 通訊作者

Received May 2023, Accepted July 2023. 2023年5月送審 2023年7月通過。

研究報告

臺灣造林地健康與萎凋相思樹土壤微生物相調查

劉則言[1)] 施欣慧[2)] 蔡佳彬[3)] 陳昭翰[1)] 吳家禎[4,5)]

摘 要

植物病原真菌*Fusarium oxysporum* f. sp. *koae*被認為是造成臺灣相思樹苗木及樹木萎凋病的原因之一。由於植物的健康被認為與土壤中的微生物族群密切相關，且*F. oxysporum*為重要的土壤傳播性病原菌之一，因此本研究嘗試分析健康與罹病植株的根圈土壤微生物相之差異性。我們採集相思樹造林地內健康和萎凋相思樹的根圈土壤，萃取土壤核酸並以次世代定序 (next generation sequencing, NGS) 技術，針對樣本中的真菌轉錄區間 (internal transcribed spacer, ITS) 和細菌16S核糖體DNA (16S ribosomal DNA, 16S rDNA) 進行定序與菌相分析。結果顯示健康與萎凋相思樹根圈土壤的真菌和細菌組成，在綱和屬的分類階層均沒有顯著的差異，且*Fusarium*屬真菌並非主要存在的微生物族群。雖然健康相思樹立地環境土壤中的真菌和細菌多樣性高於萎凋相思樹的土壤，但兩者間亦沒有顯著差異。線性判別分析 (Linear Discriminant Analysis, LDA) 發現，萎凋相思樹土壤樣本中顯著較多的微生物類群有：真菌*Cephalotrichum*屬；細菌Gammaproteobacteria和Proteobacteria門，Rhodanobacteraceae目，Kapabacteriales、Diplorickettsiales和Xanthomonadales科，*Lacunisphaera*和*Chujaibacter*屬。本研究首次對國內相思樹造林地的土壤微生物相進行分析，將有助於未來釐清與造林地相思樹萎凋有關的生物性因子並提供植物與微生物間交互作用研究之參考。

關鍵詞：*Fusarium oxysporum* f. sp. *koae*、相思樹、維管束枯萎病、土壤微生物相。

劉則言、施欣慧、蔡佳彬、陳昭翰、吳家禎。**2023**。臺灣造林地健康與萎凋相思樹土壤微生物相調查。台灣林業科學38(3):221-32。

INTRODUCTION

Acacia confusa Merr., a native tree species in Taiwan, is widely distributed in low- and mid-elevation areas. Due to its suitability for charcoal production, mushroom cultivation, furniture, and flooring panels, *A. confusa* is one of the most commonly planted trees in Taiwan. Based on the findings of the 4th National Forest Resources Survey conducted by the Forestry Bureau in 2014, the studied acacia plantation covers approximately 10,748 ha, making it the largest broadleaf tree plantation in Taiwan. Acacia trees are vulnerable to threats posed by pests and diseases. According to the "List of plant diseases in Taiwan," several diseases of acacia trees, including sooty mold, anthracnose, powdery mildew, root rot disease and damping-off disease have been recorded in Taiwan (TPS 2019).

The serious disease known as *Acacia koa* seedling wilt, caused by *Fusarium oxysporum* f. sp. *koae* f. sp. nov., was first reported in Hawaii in 1980 by Gardner (1980). That study also identified *F. oxysporum* f. sp. *koae* as a vascular fungus isolated from acacia seedlings and older branch tissues. Subsequent research conducted by Anderson et al. (2002) further

demonstrated the pathogenicity of *F. oxysporum* f. sp. *koae* on *A. koa* through greenhouse seedling inoculation experiments. In Taiwan, vascular wilt symptoms were first observed in *A. confusa* seedlings in Lioukuei District, Kaohsiung in 2018. Within the Lioukuei acacia nursery, around 60%~70% of *A. confusa* seedlings exhibited vascular wilt (Shih et al. 2020). Wilt symptoms were also observed in 2 acacia plantations in Nantou County, Taiwan in 2019. The same fungal pathogen, *F. oxysporum* f. sp. *koae*, was isolated from the infected *A. confusa*, and its pathogenicity was verified through Koch's postulates on *A. confusa* seedlings (Liu et al. 2022).

Fusarium oxysporum f. sp. *koae* belongs to the *F. oxysporum* species complex (FOSC), which comprises more than 100 host-specific strains (formae speciales) and plays many different roles in ecosystems. It is a soil saprophyte and a plant pathogen that causes vascular wilt diseases in important crops and trees (Gordon 2017). Among the different formae speciales, different virulence or pathogenesis in interactions with different host plants has been observed. These differences could be attributed to genetic variations. For example, enzymes degrading the plant cell wall were identified as important factors for successful invasion of FOSC (Roy et al. 2020). Recently, the complete genome of *F. oxysporum* f. sp. *koae* was also analyzed. Putative lineage-specific genes encoding products secreted in the xylem might be necessary for disease development in *A. koae* (Dobbs et al. 2020). Interactions between *F. oxysporum* with their hosts were also inferred through a genomic analysis, and relations between *F. oxysporum* and other microorganisms during their saprophytic or pathogenic stages were also discussed.

The community of soil microorganisms, known as the soil microbiome, is involved in many different ecological processes of the soil and is strongly related to plant health (Aislabie et al. 2013). Several studies reported that soil microbiomes play crucial roles in assisting plants in combatting various biotic and abiotic stresses during their interactions (Mendes et al. 2011, Zolla et al. 2013). However, the microbiome not only contributes to plant health but also has a negative influence on host plants (Bass et al. 2019). A study on horse chestnut bleeding canker disease raised an intriguing possibility that the bark-associated microbiota might play a key role in the spread of the disease (Koskella et al. 2017). Since *F. oxysporum* is a soil-borne plant pathogen, extensive research has unraveled the intricate interactions between this fungal pathogen and the microbiome present in the soil or within the host plant. Investigations have identified the response of both the plant rhizosphere and bulk soil microbiomes to invasion by *F. oxysporum* f. sp. *vasinfectum* in cotton. Pathogenic infection results in a consistent shift in the rhizosphere microbiome; some positive and negative microorganisms associated with the disease were also identified (Qiu et al. 2022). Another study of *Fusarium* wilt disease in watermelon also identified the rhizosphere microbiome that induces plant root resistance (Zhu et al. 2022). Moreover, a study of *Fusarium* wilt disease in tomatoes showed the potential to reconstitute the soil microbiome by combining applications of fumigation and organic amendments. This research provides us with a promising strategy for further disease control by manipulating the soil microbiome into a suppressive soil scenario (Deng et al. 2021).

Fusarium wilt diseases caused by FOSC have been extensively studied, particularly with regard to their activities against various host plants and the associated soil or host microbiomes. However, most of these studies

focused on agricultural crop plants and their respective planting environments, leaving significant gaps in understanding of the soil microbiome in acacia plantations and its relationship with vascular wilt disease of *A. confusa*. To address this research gap, we conducted a next-generation sequencing (NSG)-based investigation of the soil microbiome of an acacia plantation. Specifically, we used the bacterial 16S ribosomal DNA (16S rDNA) and the fungal internal transcribed spacer (ITS) sequencing to examine the microbial composition of collected soil samples. We also conducted a comparative analysis of the soil microbiomes of 5 wilted trees and 5 healthy trees, aiming to uncover potential associations between the soil microbiomes and acacia vascular wilt disease. To the best of our knowledge, this is the first report to comprehensively investigate the soil microbiome of acacia plantations, providing valuable insights into the microbial ecology and forest pathology of acacia vascular wilt from a multidisciplinary perspective.

MATERIALS AND METHODS

Field investigation and sampling

The acacia plantation used for field investigation and sampling was located in the Yunshui Workstation of the Chiayi Research Center, Taiwan Forestry Research Institute, Chiayi, Taiwan (23.38021°N, 120.50703°E). In a previous investigation, wilt symptoms were observed on several *A. confusa* trees where *F. oxysporum* f. sp. *koae* was isolated from vascular tissues. Five wilted trees (nos. 2-4, 13-2, 17-3, 18-4, and 18-9) in the plantation were chosen for soil collection and further soil microbiome identification. Five healthy trees (nos. 1-15, 4-8, 11-1, 18-11, and 19-8) in the same area were randomly selected as controls. We collected soil samples from around the roots (approximately 10 cm from the roots) of each tree, carefully placing them in plastic bags. The samples were then promptly transported to the laboratory at 4 °C to ensure preservation of microbial DNA.

DNA extraction and high-throughput sequencing

Microbial DNA was extracted from 0.25 g soil of each sample using the PowerSoil DNA isolation kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. For fungi, the ITS region of fungal rDNA was amplified with ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') primers (McGuire et al. 2013). For bacteria, the V3-V4 region of bacterial 16S rDNA was amplified with 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') primers (Abrahamsson et al. 2012). After polymerase chain reaction (PCR) amplification, the products were purified using the MinElute Gel Extraction kit (Qiagen). Then, a paired-end library was constructed using the Celero DNA-Seq System (Nugen, San Carlos, CA, USA). The constructed DNA libraries were then sequenced using the Illumina MiSeq platform at Tri-I Biotech (New Taipei City, Taiwan). The sequenced DNA datasets are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive database using the accession number PRJNA971944.

Data analysis

Sequences were trimmed, filtered, denoised, and merged into amplicon sequence variants (ASVs) with no chimera using DADA2 in QIIME2. The SILVA 16S rDNA gene (v132) and UNITE (v7) were the respective reference databases for all bacterial

and fungal ASV taxonomical classification. A rarefaction curve with alpha diversities based on observed ASVs (Callahan et al. 2016) was constructed in QIIME 2 (Bolyen et al. 2019). For further analysis, both the fungal and bacterial sequence datasets were rarefied to 7000 reads in each sample. Non-metric multidimensional scaling (NMDS) was analyzed based on Bray-Curtis distances using the vegan package in R. ASVs of less than 0.01% across all samples were removed. Fungal and bacterial compositions were described by grouping the ASVs into class and generic taxonomic levels. The number of ASVs belonging to a class divided by the total number of ASVs in the sample was used to estimate relative abundances. ASVs with a relative abundance of < 1% across all samples were designated as low-abundance ASVs. Relative abundance at the genus level was also estimated using the same method. Differential abundances of each microorganism between healthy and diseased plant microbiomes were calculated using an LDA effect size (LEfSe) analysis (Segata et al. 2011). These were used for high-dimensional biomarker discovery that identified genomic taxonomic characterization.

RESULTS

Field investigation

The acacia plantation in this study was established in August 2016. In our investigation, wilt symptoms of *A. confusa* in this plantation were first observed in 2019, and 23 wilted trees have been observed so far. *Fusarium oxysporum* f. sp. *koae* was isolated from all of the wilted trees (data not shown) and identified as the main pathogen. Five of the 23 wilted trees, 2-4, 13-2, 17-3, 18-4, and 18-9, were selected for a soil microbiome investigation (Fig. 1). To clarify differences in the soil microbiomes between healthy and diseased trees, soils from 5 other healthy trees, 1-15, 4-8, 11-1, 18-11, and 19-8, were

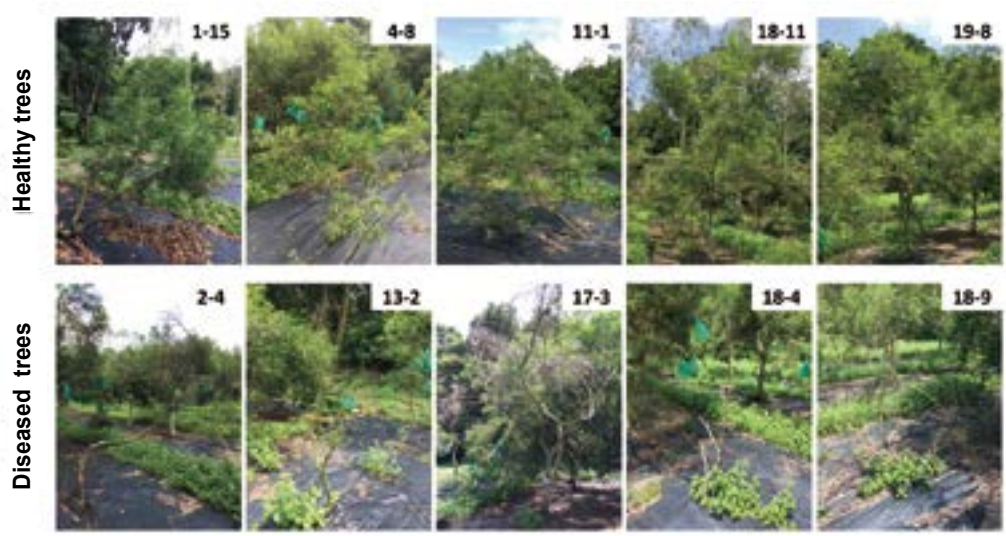

Fig. 1. Healthy and diseased *Acacia trees* in the plantation. Numbers in the top right of each figure correspond to the identification number assigned to each individual tree. The top row depicts healthy trees, while the bottom row shows diseased trees.

collected in July 2022 from the same plantation at the same time (Fig. 1).

Rarefaction curves with alpha diversity

After sequencing and raw read processing, 181,498 bacterial 16S rDNA reads (from 15,541 to 22,559 reads per sample) were derived from the 10 soil samples. In total, 251,608 fungal ITSs (from 22,220 to 28,841 reads per sample) were also derived. Refraction curves with alpha indexes (i.e., observed species, Cho1, and Shannon) were used to show that fungal and bacterial richness levels and diversities were generally saturated. While the observed species and Cho1 usually describe richness, the Shannon index describes diversity. Fungal and bacterial richness of healthy and diseased samples showed no significant differences in our investigation (Fig. 2). Fungal diversity was generally higher in healthy soil samples than in the diseased ones, but there was no significant difference between them. The same result was also obtained in the analysis of bacterial diversity.

Beta diversity

We employed non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarity distances to assess variations in microbial compositions among the samples. Surprisingly, no significant difference was observed between the healthy and diseased samples (Fig. 3). However, it is worth noting that fungal and bacterial compositions within each healthy sample exhibited considerable diversity among themselves. Fungal and bacterial composition of different diseased samples also displayed notable dissimilarities.

Microbial compositions

The ASVs were rarefied to 7000 sequences per sample to control for sampling bias and used to identify microbial composi-

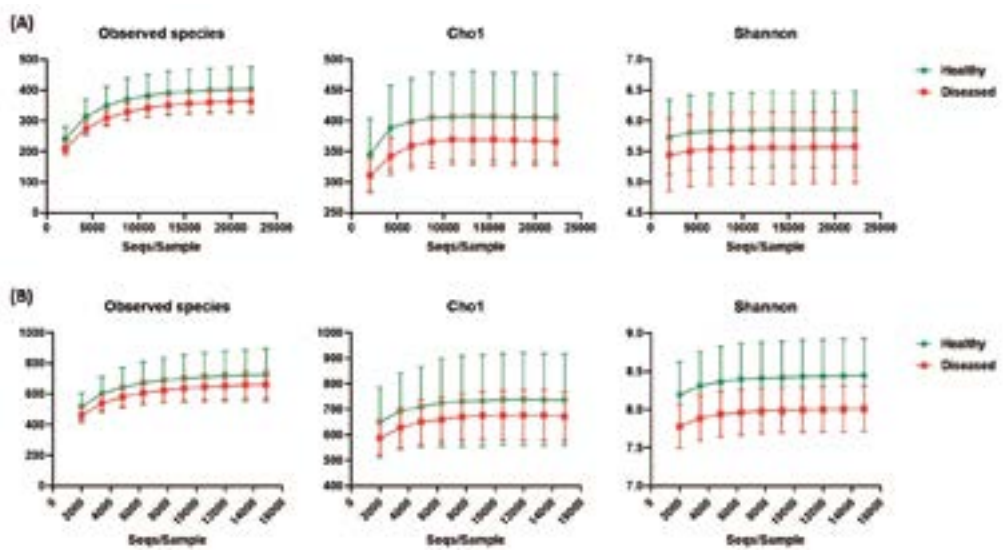

Fig. 2. Refraction curves showing alpha diversities (Observed species, Cho1, and Shannon) of the soil samples plotted for (A) fungal and (B) bacterial diversities.

tions. ASVs with percentages of < 0.01% in all samples were first removed. Then, the remaining ASVs were classified into respective taxonomic categories at the class or generic level. Percentages of each class or genus of < 0.1% were considered as low abundances. Among all of the healthy and diseased samples, the 8 most abundant fungal classes were Agaricomycetes, Dothideomycetes, Eurotiomycetes, Lecanoromycetes, Leotiomycetes, Mortierellomycetes, Rozellomycotina, and Sordariomycetes. At the taxonomic genus level, 10 fungal genera were identified as the most abundant ones: *Aspergillus, Byssochlamys, Cephalotrichum, Claussenomyces, Dictyocatenulata, Galerina, Mortierella, Paraphaeosphaeria, Talaromyces,* and *Trichoderma* (Fig. 4). However, no specific classes or genera were exclusively present in either healthy or diseased samples. For bacteria, 16 classes were identified as the abundant ones. Actinobacteria, Alphaproteobacteria, Gammaproteobacteria, and Nitrospiria were considered the 4 dominant bacterial classes among all the samples (Fig. 4). We also identified 16 dominant bacterial genera present in both healthy and diseased samples. Assessment of the bacterial analysis was similar to that of the fungal analysis, as no significant variation in bacterial communities was found in each taxonomic category between healthy and diseased samples.

Linear discriminant analysis (LDA) effect size (LEfSe) analysis

An LEfSe analysis was further conducted to identify several relatively abundant microbes in healthy and diseased samples (Fig. 5A, 5B). For fungi, the genus *Cephalotrichum* (Ascomycota: Microascales: Microascaceae) significantly dominated in diseased samples. In contrast, no taxonomic category was significantly abundant in healthy samples (Fig. 5A). For bacteria, significant abundance var-

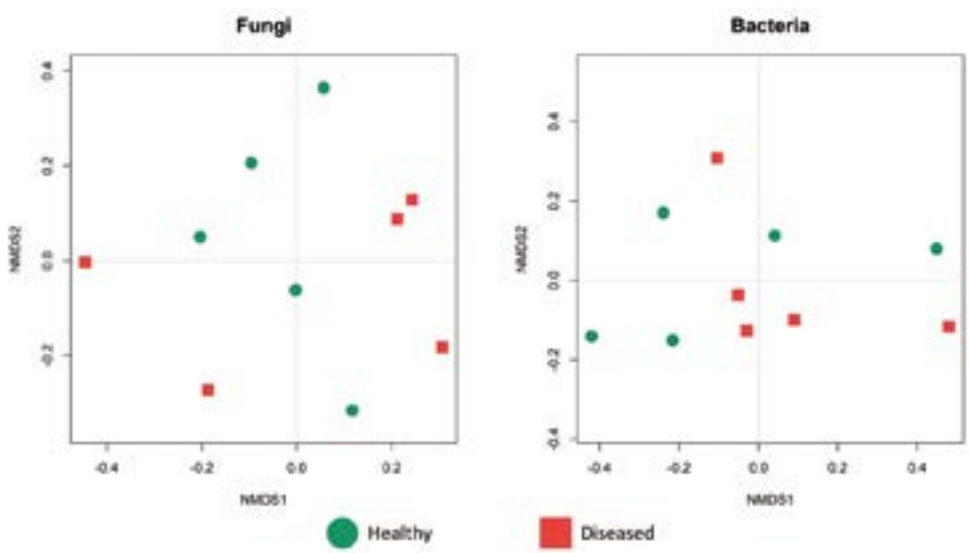

Fig. 3. Non-metric multidimensional scaling (NMDS) was performed to visualize (A) fungal and (B) bacterial community structures in the soil samples collected from both healthy and diseased trees. Red squares represent diseased trees, green dots represent healthy trees.

ied for healthy and diseased samples. Among healthy samples, 1 phylum (Armatimonadota), 5 orders (Acidobacteriales, Gemmatales, Pseudonocardiales, Solirubrobacterales and Streptosporangiales), 6 families (Xanthobacteraceae, Nitrosomonadaceae, Gemmataceae, Pseudonocardiceae, Chthonomonadaceae and Thermomonosporaceae), and 4 genera (*Actinomadura, Crossiella, Chthonomonas, Polycyclovorans*) were significantly more abundant. Contrastingly, in diseased soils, 1 phylum (Proteobacteria), 2 classes (Gammaproteobacteria and Kapabacteria), 3 orders (Kapabacteriales, Diplorickettsiales and Xan-

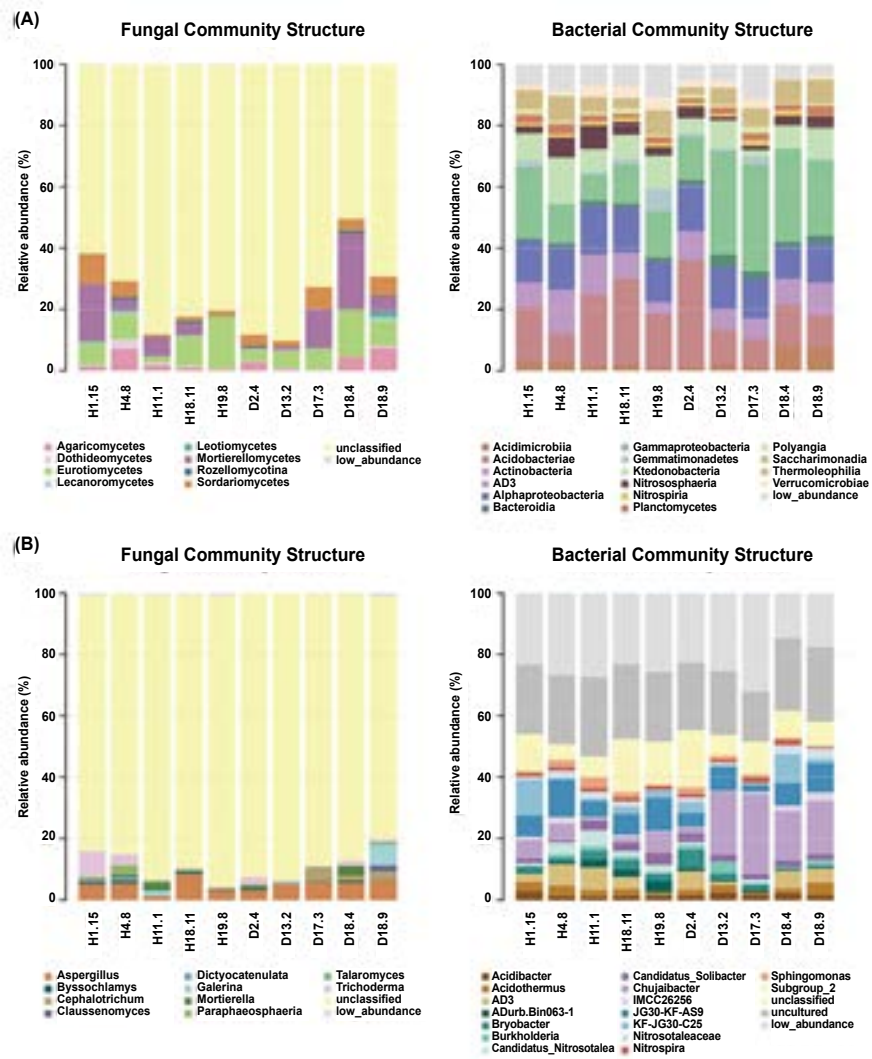

Fig. 4. Relative abundances of microbial compositions in each soil sample. Healthy samples are labeled H1.15, H4.8, H11.1, H18.11, and H19.8, while diseased ones are labeled D2.4, D13.2, D17.3, D18.4, and D18.9 on the horizontal axis. Fungal and bacterial compositions were represented at the class level in A and at the generic level in B.

thomonadales), 2 families (Rhodanobacteraceae and Diplorickettsiaceae), and 2 genera (Chujaibacter and Lacunisphaera) were significantly more abundant (Fig. 5B).

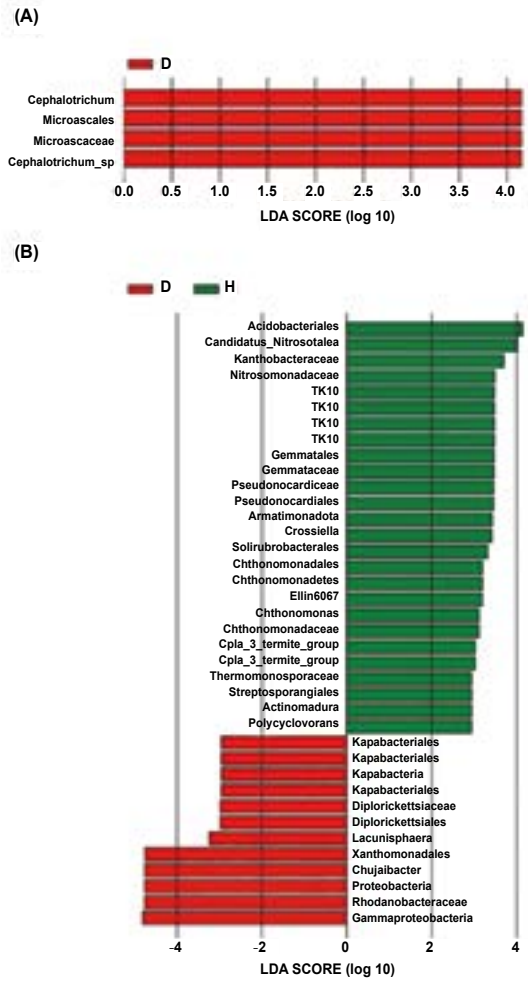

Fig. 5. Linear discriminant analysis (LDA) effect size (LEfSe) analysis was performed on relative abundance data of microbial communities in soil samples collected from healthy trees (*n*=5) and diseased trees (*n*=5). Abundant fungal community in diseased samples is presented in A, and abundant bacterial communities in both healthy and diseased samples are presented in B.

DISCUSSION

Our study aimed to propose a novel approach to identifying biotic factors related to vascular wilt disease in acacia plantations. Although *F. oxysporum* f. sp. *koae,* a soilborne fungus, is commonly believed to be the pathogen responsible for this disease, our study focused on investigating all soil microorganisms and identifying their associations with the disease. This is the first investigation of the soil microbiome of an acacia plantation in central Taiwan using NGS. Unlike traditional plant disease investigation methods, NGS provides comprehensive and detailed information about the bacterial and fungal compositions of soil samples.

Wilted *A. confusa* trees infected with *F. oxysporum* f. sp. *koae,* appear randomly throughout Taiwanese plantations, but a definitive center of the disease could not be identified. Moreover, no specific pattern was observed regarding the timing of wilt symptoms for each affected tree. Variations in wilted tree distributions and timing of symptoms might be indicative of differences in disease resistance of individual trees (Anderson et al. 2002, Naidoo et al. 2019). It is also possible that some other biotic or abiotic agents present in the plantation or in other wilt-infected plant species near the plantation could also be contributing to the occurrence of acacia vascular wilt disease (Anderson et al. 2002, Shih et al. 2020, Liu et al. 2022).

We also investigated the potential role of the soil microbiome as a biotic agent associated with acacia vascular wilt disease. However, our NGS analysis did not identify any ASVs corresponding to the fungal genus *Fusarium* in any of the soil samples collected from the acacia plantation, regardless of tree health. Given that the primers employed in this study are commonly utilized for soil

and plant microbiome analysis, the absence of *Fusarium* spp. should not solely be attributed to sequencing biases and preferences of NGS methods (Qiu et al. 2020). While it is generally known that plant pathogenic fungi can directly affect the microbiome within the same ecological niche and become a dominant group, our observations revealed no significant differences in either fungal or bacterial populations when comparing microbial communities of healthy and diseased soils. This contrasts with previous studies, which reported that both *F. oxysporum* and *Phellinus noxius* can alter the microbial community structure of diseased specimens (Tsang et al. 2020, Qiu et al. 2022). However, those studies mainly focused on the rhizosphere or plant tissues as the ecological niches for observing microbial changes. Different time and plant compartments of sample collection might also be a limiting factor for plant microbiome investigations. To overcome this challenge, a more-comprehensive methodology that takes into account differentiation of various disease stages and plant compartments should be used (McPherson et al. 2018). Our results suggest that the soil microbiome might not be directly associated with the occurrence of acacia wilt disease, although further investigation is required to confirm this preliminary hypothesis. We propose that a more-comprehensive investigation of the microbiome of acacia plantations is necessary to fully understand the potential role of the soil microbiome in the occurrence of acacia wilt disease. This may involve examining the microbial communities in different compartments of trees, such as the roots, stems, and leaves, as well as investigating the microbiome under different disease status and weather conditions. Additionally, further studies could focus on examining interactions between microbial communities and environmental factors that may influence the development of plant diseases. To sum up, a more-detailed investigation of the microbiome of acacia plantations could provide insights into the biotic agents associated with acacia wilt disease and inform strategies for future disease management.

The present study investigated the soil microbiome of an acacia plantation and compared microbial diversity between healthy and diseased soil samples. Despite the lack of statistically significant differences in the results, the observed differences in diversity between healthy and diseased samples were consistent with previous findings on asymptomatic and symptomatic Norway spruce trees (Kovalchuk et al. 2018) and trees affected by brown root rot disease (Tsang et al. 2020). Several factors, including disease type, plant compartment, and host plant species, may influence variations in microbial diversity (Kovalchuk et al. 2018, Tsang et al. 2020). Moreover, the microbiome diversity in the diseased compartments of trees is generally lower than that of healthy ones, especially in regions impacted by pathogen invasion and colonization. It is worth noting that the LEfSe revealed differential abundant microbial communities in both healthy and diseased samples, even though these groups might not constitute the dominant fungal or bacterial populations. While differential taxonomic groups were identified, a majority of them could not be directly associated with any known information regarding acacia wilt disease or host plant resistance. Therefore, further studies are necessary to elucidate the relationship between these microbial communities and acacia wilt disease.

In this study, the potential role of the soil microbiome in acacia vascular wilt disease was examined, and the microbial diversities and differential abundances of microbial groups of healthy and diseased soil samples

were analyzed. Results inferred that the soil microbiome might not be directly linked with the occurrence of acacia wilt disease, but further research is necessary to confirm this preliminary inference. The slight differences observed in microbial diversity between healthy and diseased samples were consistent with previous research (Kovalchuk et al. 2018, Tsang et al. 2020). This study provides invaluable insights into the biotic agents intricately associated with acacia wilt disease, highlighting the critical importance of conducting comprehensive and exhaustive investigations of the microbiome within acacia plantations. Furthermore, it highlights the need to understand the various ecological factors that influence the composition and dynamics of the microbiome in order to effectively manage and mitigate the impacts of acacia wilt disease.

CONCLUSIONS

This is the first study to investigate the soil microbiome in an *A. confusa* plantation in Taiwan using NGS technology. By comparing microbial communities in soils surrounding healthy and diseased trees, we aimed to identify potential soil microbial factors that may impact the health of *A. confusa*. While the pathogenic fungus *F. oxysporum* f. sp. *koae* was not identified in the analyzed soil microbiome, it cannot be excluded that it may still be present in the soil and contribute to the occurrence of wilted *A. confusa* through soil transmission. Varying microbial diversities and significant microbial communities were still indicated through comparing healthy and diseased samples. As a preliminary investigation of the soil microbiome in *A. confusa* plantations in Taiwan, we provide basic information on the existence of microorganisms. Our study can serve as a basis for further studies on vascular wilt of *A. confusa* and the soil microbiome of *A. confusa* plantations.

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