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Research Article

Co-occurrence pattern of bacteria and fungi on the leaves of the invasive aquatic plant *Alternanthera philoxeroides*

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Abstract

The microbes that are attached to aquatic plants play critical roles in nutrient cycles and the maintenance of water quality. However, their community compositions, biodiversity and functions have not been well explored for the invasive plants in inland waters. Here, the co-occurrence patterns between bacteria and fungi on the leaves of *Alternanthera philoxeroides* and their potential ecological interactions were studied during the growing seasons. Along with significant variations in the alpha diversity of attached microbes over time, shifts in their community composition were significantly associated with the dynamics of plant stoichiometry, substrate composition and extracellular enzyme activity. Deterministic processes (heterogenous selection) play a predominant role in community assembly of the attached bacteria, while stochasticity (undominated process) was the major driver for the attached fungal assembly. Compared with the free-living microbial network, the attached microbial network was structurally simple but highly modular. The attached microbes had more intra-phylum links (primarily within the phyla *Actinomycetota*, *Alphaproteobacteria*, *Bacillota* and *Basidiomycota*) and distinct co-exclusion patterns between bacteria and fungi in the modules. In summary, the study will be helpful in understanding the microbes and their interactions in the phyllosphere of *A. philoxeroides*, an key invasive species under national management and control.

Keywords: co-occurrence networks, community assembly, invasive macrophyte, phyllospheric microorganisms

Introduction

Macrophytes are widely distributed in aquatic ecosystems, and they play important roles in the structure and function of shallow aquatic habitats. The leaves of macrophytes provide substrates for microbes to attach and grow, and they have special niches. The phyllosphere represents the substrate surface of plants and is a special niche that harbors diverse species of microbes (Xie et al. 2015, Vacher et al. 2016). Attached bacteria that have colonized the phyllosphere can influence plant growth and fitness (Vorholt 2012, Kembel et al. 2014), and fungi that have attached can contribute to the decomposition of leaf litter and play an important role in recycling carbon and nutrients in ecosystems (Yao et al. 2019). The attached microbes form biofilms in aquatic ecosystems and play a major role in regulating the nutrient cycle and energy flow in water bodies. There is increasing research interest in utilizing natural periphytic biofilms in wastewater treatment, non-point source pollution control and the remediation of polluted waters (Yan et al. 2019). Multiple studies of bacteria and fungi attached to leaves have primarily focused on terrestrial plants, such as *Arabidopsis thaliana* (Bai et al. 2015) and the red-osier dogwood (*Cornus stolonifera*) (Osono 2007). However, very few studies have addressed the microbial diversity of the phyllosphere in aquatic environments.

Alternanthera philoxeroides (Mart.) Griseb is a perennial herbaceous plant that is both stoloniferous and amphibious. The native range of this species is thought to be the Parana River region of southern Brazil, Paraguay and Argentina, and this species has spread in tropical, subtropical and warm temperate regions, including China, USA, Australia, New Zealand, Indonesia, India and Thailand (Yan et al. 2020). In China, *A. philoxeroides* was first introduced into suburban Shanghai from Japan as horse feed in the late 1930s, and it has since invaded large areas south of the Yellow River Basin and can be found sporadically in northern China (Wang et al. 2005). The aquatic type of *A. philoxeroides* grows rapidly in the aquatic environment, covers the water area, decreases the biomass of native plant species, alters the patterns of nutrient cycling, blocks drainage and irrigation canals, affects the freshwater culture and farmland irrigation and causes economic and ecological problems in the areas invaded (Ding and Zhang 2014, Fan et al. 2016). Therefore, the control and prevention of *A. philoxeroides* is of great significance for maintaining the biodiversity, ecosystem and social economy of the habitat that has been invaded (Yan et al. 2020).

Some previous studies indicated that the attached bacteria are more diverse and form a distinct community composition compared with the free-living bacteria from the surrounding water

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(He et al. 2014). Free-living bacteria have been regarded as a major seed bank for the attached bacteria, which has an important influence on the assemblage of attached bacteria (Bellés-Garulera et al. 2016). Macrophytes secrete and provide nutrients and oxygen to the attached bacterial communities, which facilitates their growth (Zhang et al. 2021a) . Attached microbes can protect macrophytes from external hazards, such as pernicious microbes and invertebrate species (Hashidoko 2005). Plant–fungal associations are frequently key drivers of the success of plant invasion. The attached fungi can benefit their invasive hosts by enhancing growth promotion and disease resistance and enhancing the tolerance of environmental stress. However, the roles of attached fungi can vary when a given invasive plant faces different stresses (Fang et al. 2021). However, few studies have directly compared the diversity of attached and free-living fungi in the surrounding water.

Many previous studies only focused on the diversity and community structure of microbial communities, but there has been less attention on the direct or indirect interactions between the microbial taxa that coexist in the environmental samples (Barberán et al. 2012). The exploration of potential interactions between microbial taxa (bacteria–bacteria, fungi–fungi and bacteria–fungi) in complex and different microbial communities can help to elucidate the environmental niches of microbes and their potential functions (Ruan et al. 2006, Chaffron et al. 2010). Co-occurrence/co-exclusion patterns indicate mutualism or predation, respectively, and the competition between species can be identified in a network analysis (Deng et al. 2012). In addition, network analyses have been used to study bacterial communities in soil, freshwater lakes and activated sludge (Zhou et al. 2011, Barberán et al. 2012, Ju et al. 2014, Zhao et al. 2016) and fungal communities in soil (He et al. 2017, Xiong et al. 2021). Few studies have analyzed the networks between bacteria and fungi.

In this study, we investigated the attached microbes on *A. philoxeroides* (A: both bacteria and fungi) throughout the growing season and the free-living microbes in the surrounding water (F: both bacteria and fungi). *Alternanthera philoxeroides* is an invasive plant that can grow in a variety of habitats, including dry land. However, it is usually found in water. Alien plant invasion in native plant communities will decrease the aboveground biodiversity and increase the dominance of invasive species (Rusterholz et al. 2017). Therefore, biological invasion usually changes the composition of species and ecosystem structure through the inhibition and exclusion of the native species (Liu et al. 2020). The goal of this study was to determine: (i) How did the composition of bacterial and fungal communities change during the growing season of *A. philoxeroides?* (ii) What was the community assembly mechanism for the phyllospheric microorganisms? and (iii) How did bacteria and fungi interact in the phyllosphere of *A. philoxeroides?* Illumina NovaSeq 6000 (Illumina, USA)-based sequencing was used to study the composition and dynamics of bacterial and fungal communities during the growing season. Our results will help to better understand the ecology of attached microbes and the free-living microbes in aquatic ecosystems.

Materials and methods

Experimental design and sample collection

In May, June, July, August, September and October 2015, *A. philoxeroides* plants were collected from a site (N32°4'14.88', E120°39'46.80') in a freshwater lake in Nantong, China. Samples were taken throughout the entire growing season of the plant, including the early stage (May and June), middle stage (July, August and September) and late stage (October) (Fig. 1). Three replicate plants were collected by hands that wore sterile gloves. Each plant sample was stored in an aseptic plastic bag. Moreover, the surrounding water was also sampled to study the variation of composition of the planktonic microbial community. Approximately 1 L of water surrounding the plant at the sampling site was collected using an aseptic plastic bottle. Approximately 500 ml of water was used for chemical analyses, and the other 500 ml was used to analyze the community composition of planktonic microorganisms. The concentrations of total nitrogen (TN) and total phosphorus (TP) of the water were measured as previously described (Greenberg et al. 1992). The total organic carbon (TOC) was analyzed using a Torch TOC Analyzer (Teledyne Tekmar, Mason, OH, USA) using high temperature catalytic oxidation. All the plants and water samples were placed on ice and kept cool before their transfer to the laboratory as soon as possible (< 4 h).

Characteristics of *A. Philoxeroides* and the assays of hydrolase activity

Approximately 5 g of leaves was freeze-dried to a constant weight and ground to a homogeneous fine powder. The carbon content (C%) and nitrogen content (N%) of the freeze-dried leaf powders were analyzed by an integrated oxidation and detection device (Leco CN2000, LECO Corp., St Joseph, MI, USA). The phosphorous content (P%) was measured using inductively coupled plasma-optical emission spectroscopy (model: MIC IC, Metrohm, Switzerland) as described by Henschler (1988). The contents of cellulose, hemicellulose and lignin were measured using gel permeation chromatography (Waters 1515–2414; Waters, Milford, MA, USA) and a UV spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan) using the NREL method as previously described (Sluiter *et al.* 2008).

A total of 1 g of plant leaves was mixed with 9 ml of 10 mM PBS buffer (pH 8.0) and incubated on ice for 15 min. The homogenate was centrifuged at 1510 × g for 20 min. The liquid supernatants were used to measure the activities of cellulase, xylanase, polyphenol oxidase and peroxidase, which were determined using ELISA kits according to the manufacturer's instructions (Shanghai Shuangying Biological Technology Co., Ltd., Shanghai, China).

DNA extraction, PCR amplification, sequencing and raw data treatment

A total of 2 g of leaves was transferred to a sterile 50 ml polyethylene tube that contained 40 ml of 2 mM PBS with 0.01% (v/v) Tween 80. The attached microbes were removed from the leaves using ultrasonication for 5 min after 30 s of shaking (10 × g). The suspension that contained both the attached and free-living microbes was collected by filtration through 0.2 μm pore polycarbonate filters and stored at −80°C until the nucleic acids were extracted. After the filters were cut into small pieces, the genomic DNA was extracted using an E.Z.N.A. water DNA kit D5525-01 (Omega BioTek, Norcross, GA, USA). The V3-V4 region of bacterial 16S rRNA genes was amplified from the genomic DNA using the primer pairs 341F (5′-CCTACGGGNGGCWGCAG) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (Herlemann et al. 2011), while the V4 region of fungal 18S rRNA genes was amplified using the primer pairs 565f (5′-CCAGCASCYGCGGTAATTCC-3′) and 964b (5′-ACTTTCGTTCTTGATYRA-3′) (Zorz et al. 2019).

The raw reads of the 16S and 18S rRNA gene sequences were processed using the Mothur program (V. 1.30.0, http://www.mothur.org, 2013) according to the NovaSeq 6000 standard operating procedure. The raw reads were combined, denoised, trimmed,

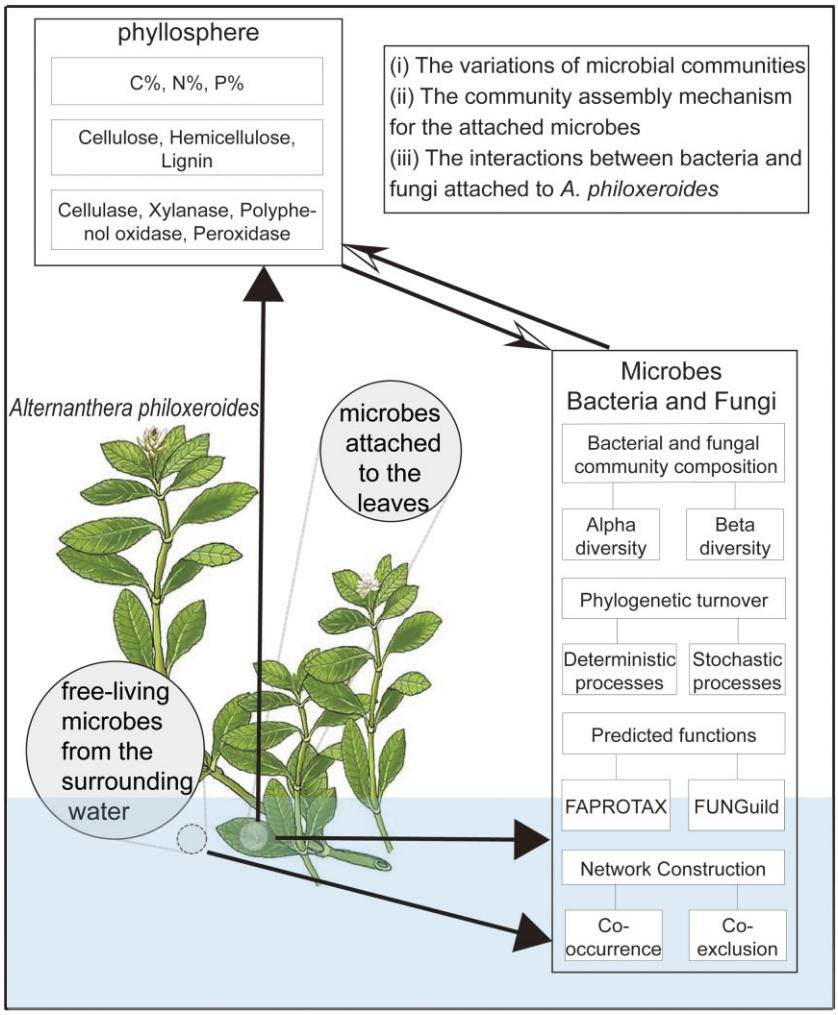

Figure 1. Experimental design to investigate the co-occurrence pattern of bacteria and fungi on the leaves of *Alternanthera philoxeroides* during the growing seasons.

quality filtered and finally aligned to databases. The 16S sequences were aligned using the Ribosomal Database Project (http://rdp.cme.msu.edu/) and the 18S sequences were aligned to the SILVA database (http://www.arb-silva.de) (Quast et al. 2013). In this study, we avoided the dangers of closed-database operational taxonomic units (OTUs) clustering and instead demonstrated a new method that resolves amplicon sequence variants (ASVs) from Illumina-scale amplicon data. ASVs can sufficiently control errors down to the level of single nucleotide differences over the gene region that has been sequenced (Callahan et al. 2017). ASV methods infer the biological sequences in the sample prior to the introduction of amplification and sequencing errors, and distinguish sequence variants differing by as little as one nucleotide (Callahan et al. 2017). In other words, the quality sequences are clustered into ASVs at 100% similarity level.

Diversity estimations of microbial communities

The alpha diversity indices were estimated using the Mothur program (Lozupone et al., 2011; Jiang et al., 2013). The species richness based on species (ASV) distribution and Faith's phylogenetic diversity (PD) index (Faith 1992) based on phylogeny were calculated. The Bray-Curtis dissimilarity based on the ASV table was calculated as beta diversity. The dissimilarities in the microbial community composition were visualized using a non-metric multidimensional scaling (NMDS) plot. Beta diversity was estimated and the NMDS plots were performed using the vegan package in R.

Phylogenetic turnover and the quantification of various ecological processes

The ecological processes governing microbial community assembly include homogeneous selection (i.e. selection under similar biotic and abiotic environmental conditions), heterogeneous selection (i.e. selection under variable biotic and abiotic environmental conditions), homogenizing dispersal, dispersal limitation and undominated processes (Stegen et al. 2013, 2015). In this study, to estimate the relative contributions of various ecological processes, a null model-based quantitative framework proposed by Stegen et al. (2013) was used, in which null model-based phylogenetic and taxonomic β-diversity metrics (i.e. β-nearest taxon index [βNTI] and the modified Bray–Curtis-based Raup–Crick [RC_{Bray}]) were calculated. The βNTI was calculated using the function *bNTI.p* with 1000 randomizations in the R package *ieggr* (v. 2.9) (Ning and Escalas 2017) to measure phylogenetic turnover in community composition between pairwise samples. RC_{Bray} was calculated using the function *RC.p* with 1000 randomizations in the R package *ieggr* (Chase et al. 2011). Here, the null model analysis was run

separately for each habitat type and season. The fraction of pairwise comparisons with βNTI values and RC_{Bray} values was then applied to quantify the relative contributions of ecological assembly processes (the methods are detailed in the supplementary materials).

Network construction and characterization

The Hmisc package in R was used to calculate the correlation matrix (*R* value) and the significance matrix (*P* value) by counting all the possible pairwise Spearman's rank correlations among all the selected ASVs of each group. Only strong correlations were selected (i.e. those with statistically significant, $P < 0.05$, Spearman's correlation coefficient > 0.8 [or < -0.8]) to complete the network analysis (Ruan et al. 2006). The q-value package in R was used to calculate the Q-value, which represents the fraction of false positives or false negatives if a given pair is identified to be significant (Q-values < 0.05) (Ruan et al. 2006). The important information, including nodes (ASVs), edges (interactions), modules, weights and positive or negative correlations, were imported into Gephi v. 0.9.2, and topological networks were generated and visualized in Gephi v. 0.9.2 (Bastian and Heymann 2009). The network complexity was calculated as linkage density (links per OTU) among bacteria, fungi, fungi–bacterial only or all fungal and bacteria OTUs (Wagg et al. 2019).

Statistical analysis

A principal co-ordinates analysis (PCoA) and redundancy analysis (RDA) were performed using the package vegan and ggplot2 in R. The coordinates of PCo1 and PCo2 were obtained based on the PCoA of the free-living microbes. All the plant physiological characteristics and the PCo1 and PCo2 of free-living microbial community were used as the influence factors in the RDA. The relationships between alpha diversity of the microbial community composition and plant physiological factors were investigated using a nonlinear regression, and a general linear regression was used to investigate the relationships between beta diversity of the microbial community composition and the Euclidean distance of plant physiological characteristics. Nonlinear and linear regressions were generated using OriginPro 2022 (OriginLab, Northampton, MA, USA).

Data accession numbers

All the sequenced raw data from the DNA of bacteria and fungi that were attached to *A. philoxeroides* and those that were free-living raw data were uploaded to the National Centre for Biotechnology Information (NCBI) sequence reads archive (SRA) and can be accessed with the BioSample numbers SAMN30863573-SAMN30863608 and SAMN30863609-SAMN30863644 for the bacteria and fungi, respectively.

Results

Variations in the bacterial community composition during the growing season

In total, 3341 375 high-quality bacterial sequences that ranged from 40 440 to 245 281 in different samples with a median length of 400 bases were obtained. After rarefaction to the minimum reads number, 40 440, and removing singletons, 7220 ASVs were detected from all the sequences with a range of 766 to 1894 ASVs for each sample.

The alpha indices, including the species richness and PD, of both the bacteria attached to *A. philoxeroides* and those that were free-living, differed significantly as the growing season progressed ($P < 0.01$). The alpha diversity of the attached bacteria failed to fit the change curve (FitPolynomial, $P > 0.05$), while the free-living bacteria increased in the early stage and then decreased during the growing stages ($P < 0.001$) (Fig. 2a). Moreover, there was no significant difference in alpha diversity between the attached and free-living bacteria (permutation-based *t*-tests, $P > 0.05$). We observed that the attached bacterial communities differed significantly from the free-living bacterial communities in community compositions (ANOSIM: $R = 0.937$, $P = 0.001$). The NMDS plots derived from the Bray-Curtis dissimilarities indicated that the lifestyle of bacteria had an influence on the community composition of attached and free-living bacteria (Fig. 2b).

There was a significant divergence of the relative abundances of genera between the attached and free-living bacteria (Adonis, df = 1, $F = 23.44$, $P = 0.001$) (Fig. 2c). *Sphingomonas* (*Actinomycetota*), *Methylobacterium* (*Alphaproteobacteria*), *Pantoea* (*Gammaproteobacteria*), *Exiguobacterium* (*Bacilli*), *Massilia* (*Betaproteobacteria*), *Bacillus* (*Bacilli*), *Curtobacterium* (*Actinomycetota*), *Pseudomonas* (*Gammaproteobacteria*), *Chryseobacterium* (*Flavobacteriia*), *Hymenobacter* (*Cytophagia*), *Aquabacterium* (*Betaproteobacteria*), *Xanthobacter* (*Alphaproteobacteria*), *Pseudacidovorax* (*Betaproteobacteria*), *Rhizobium* (*Alphaproteobacteria*) and *Aureimonas* (*Alphaproteobacteria*) were more abundant in the attached bacterial communities ($P < 0.05$). *Rhodococcus* (*Actinomycetota*), unassigned *Acidimicrobiaceae* (*Actinomycetota*), *GpIIa* (*Cyanobacteria*) and *Ilumatobacter* (*Actinomycetota*) were more abundant in the free-living bacterial communities ($P < 0.001$). The ASVs from surrounding water comprised a portion of the attached bacterial community (Fig. S1a).

Variations in the fungal community composition during the growing season

In total, 1762 362 high-quality fungal sequences that ranged from 647 to 160 264 in different samples with a median length of 380 bases were obtained. After rarefaction (1000) and removing singletons, 145 ASVs were detected from all the sequences with a range of 11–42 ASVs for each sample.

The alpha-indices of both the attached and free-living fungi differed significantly during the growing season ($P < 0.01$). The alpha diversity of the attached fungi decreased in the early and middle stages and increased in the late stage (FitPolynomial, $P < 0.01$), while the free-living fungi failed to fit the change curve ($P > 0.05$) (Fig. 3a). Moreover, there was no significant difference in the alpha diversity between the attached and free-living fungi (permutation-based *t*-tests, $P > 0.05$). There were significant differences between the community compositions of attached and free-living fungi (ANOSIM: $R = 0.467$, $P = 0.001$). The NMDS plots derived from the Bray-Curtis dissimilarities of fungal community composition indicate that the fungal community differed along with the lifestyle of fungi (Fig. 3b).

There was a significant divergence in the relative abundances of phyla between the attached and free-living fungi (Adonis, df = 1, $F = 28.83$ $P = 0.001$) (Fig. 3c). *Basidiomycota* was more abundant in the attached fungal communities (mean, 95.25% vs 55.28%, permutation-based *t*-test, $P = 0.001$). *Chytridiomycota* was more abundant in the free-living fungal communities (mean 18.59% vs 0.55%). *Ascomycota* was found from samples of both the attached fungi (mean, 3.64%) and free-living fungi (mean, 8.93%). The ASVs from surrounding water were attributed to a small portion of the attached fungal community (Fig. S1b).

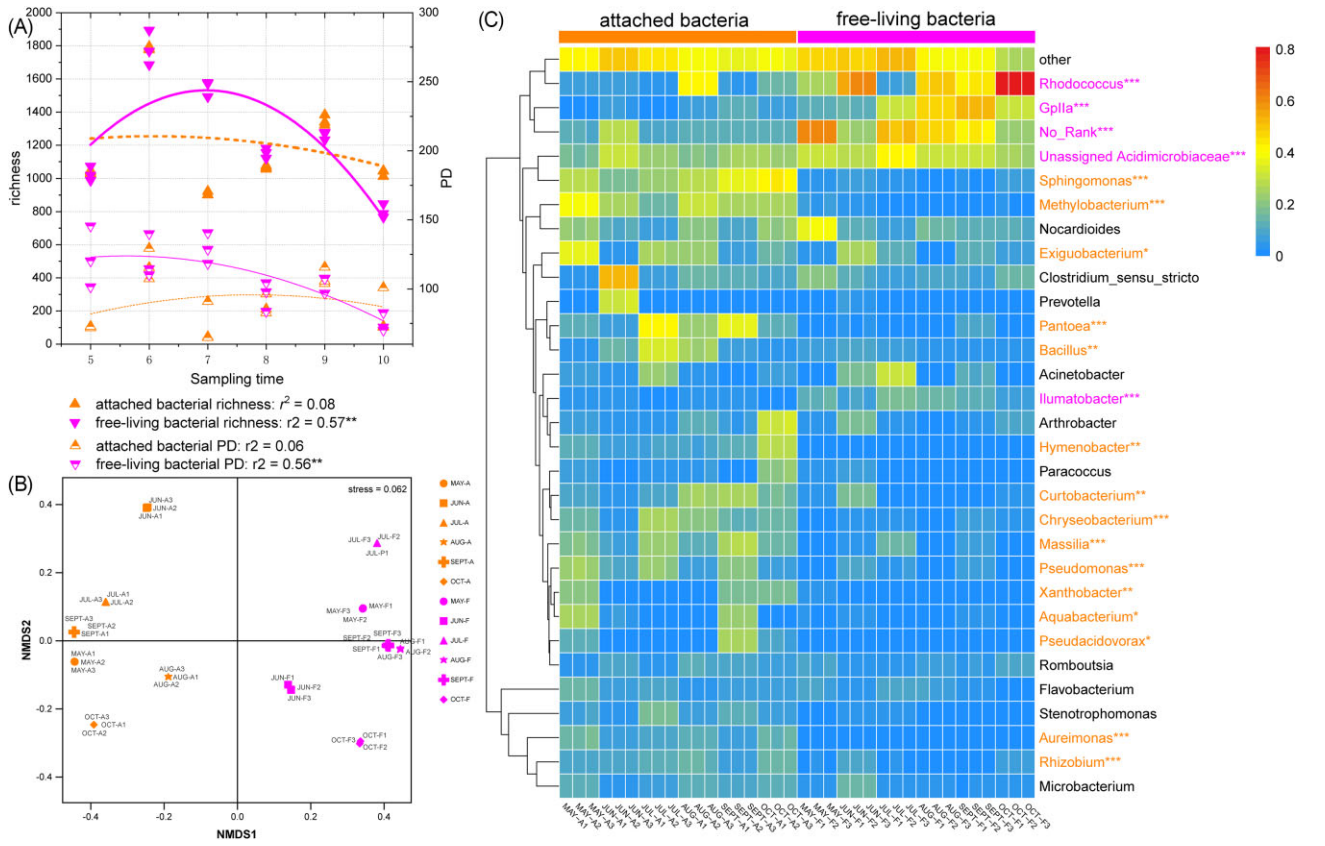

Figure 2. Alpha diversity, which included the species richness and phylogenetic diversity (PD) of attached and free-living bacterial communities in the growing seasons (**A**), non-metric multidimensional scaling (NMDS) plots derived from incidence-based Bray-Curtis dissimilarities of attached and free-living bacteria (**B**) and a heatmap of different bacterial genera between the attached and free-living bacterial community in the growing seasons (**C**). The color bar represents the relative abundance of each genus, and the relative abundance was transformed by Hellinger. A STAMP analysis of the relative abundance of the bacteria between the attached and free-living groups provided statistical significance. $*P \leq 0.05$. $**P \leq 0.01$. $***P \leq 0.001$. Genera with significantly higher relative abundances in attached bacteria are marked orange, while those with significantly higher relative abundance in free-living bacteria are marked magenta.

Ecological processes regulating the assembly of microbial communities

Two metrics (i.e. βNTI and RC_{Bray}) based on null model analysis were used to estimate the relative contributions of various ecological processes at different times. The results suggested that the deterministic process (almost exclusively heterogeneous selection) was predominant in regulating the community assembly in attached bacteria (Fig. 4a). The stochastic processes were slightly more important than the deterministic process for the community assembly of free-living bacteria (Fig. 4b). Generally, stochastic processes were most important in fungi community assemblies, for not only attached but also free-living fungi, with the distinct contribution of undominated process (61.44% and 65.36%, respectively, Fig. 4c and d). By comparison, heterogeneous selection was more important in governing the assembly of attached fungi (25.49%) than free-living fungi (8.50%) among the deterministic processes. The dispersal limitation made a large contribution to the assembly of both free-living bacterial and fungal communities in surrounding water (47.06% and 22.22%, respectively) than attached niche. Homogenizing dispersal (3.27%–6.54%; Fig. 4) and homogeneous selection (0%–5.88%; Fig. 4) contributed less fraction to the assembly processes of all the microbes.

Relationships between the attached microbial community, material composition and enzyme activities on *A. Philoxeroides*

The contents of nutrients, compositions of material and activities of enzymes except for xylanase differed significantly during the growing seasons (PERMANOVA, df = 5, n = 18, $P < 0.001$). The contents of nutrients and cellulose and the activity of peroxidase increased at first and then decreased with time, while the content of hemicellulose and activity of polyphenol oxidase decreased at first and then increased.

The results from the RDA indicated that the PCo1 of free-living bacteria and fungi in the ambient water significantly correlated with the compositions of the attached bacterial and fungal community, respectively ($P < 0.05$) (Fig. 5). The C%, P% and peroxidase activity of *A. philoxeroides* only significantly correlated with the compositions of bacterial community ($P < 0.05$). The N/P ratio, hemicellulose, lignin and the activity of xylanase were significantly related to the fungal community compositions during the decomposition process ($P < 0.05$). The factors that drove the dynamics of the microbial communities varied among the different months (Fig. 5a and b). In May, the hemicellulose of *A. philoxeroides* significantly affected the fungal community composition. In June, the C% and P% of *A. philoxeroides* af-

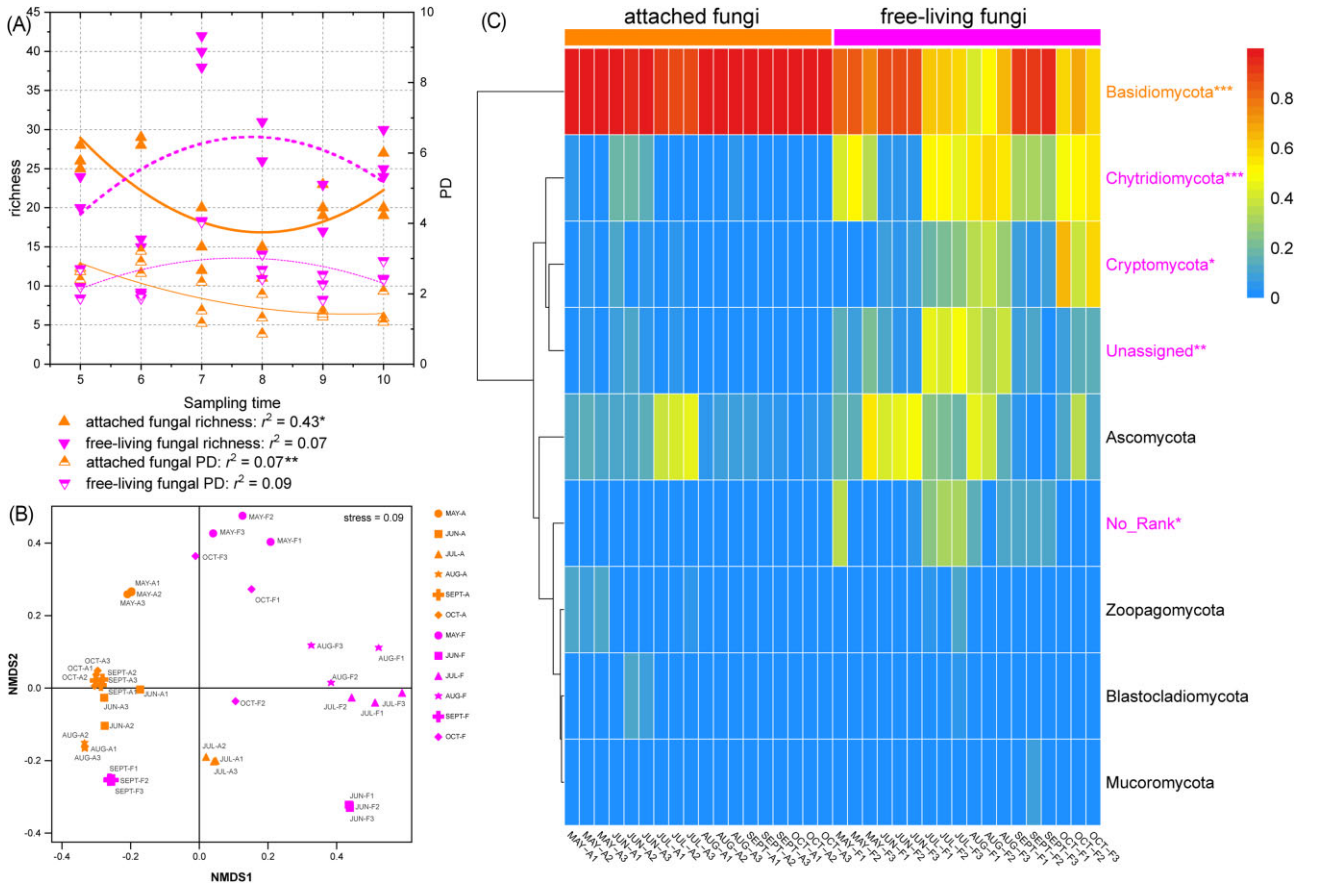

Figure 3. Alpha diversity, including species richness and phylogenetic diversity (PD) of attached and free-living fungal communities in the growing seasons (**A**), non-metric multidimensional scaling (NMDS) plots derived from incidence-based Bray-Curtis dissimilarities of attached and free-living fungi (**B**) and a heatmap of different fungal phyla between the attached and free-living fungal community in the growing seasons (**C**). The color bar represents the relative abundance of each phylum, and the relative abundance was transformed by Hellinger. A STAMP analysis of the relative abundance of the bacteria between the attached and free-living groups was used to show the statistical significance. $*P \leq 0.05$. $**P \leq 0.01$. $***P \leq 0.001$. Phyla with significantly higher relative abundances in attached fungi are marked orange, while those with significantly higher relative abundance in free-living fungi are marked magenta.

fected the bacteria, while the N/P ratio and the xylanase activity correlated with the fungal community. In July and September, the peroxidase activity and PCo1 obviously synchronized with the dynamics of the bacterial community, while the lignin and PCo1 correlated with the fungal community in September and October.

A nonlinear fitting model was applied to the attached bacterial and fungal alpha diversity with the physiological characteristics of the types of litter. The bacterial PD nonlinearly correlated with the N/P ratio ($P < 0.05$), while the fungal richness and PD nonlinearly correlated with the hemicellulose and C/P ratio ($P < 0.01$ and $P < 0.05$, respectively). A linear fitting model was applied to the bacterial and fungal dissimilarity with the Euclidean distance of plant physiological characteristics (Fig. S2). Typically, the bacteria had factors that correlated more closely than those of the fungi. The bacterial community dynamics linearly correlated with N%, P%, stoichiometry, cellulose, hemicellulose and activities of the enzymes except for xylanase. By contrast, the P%, N/P ratio and all the material compositions and enzyme activities were identified to correlate with the fungal communities during the growing season.

Co-occurrence pattern of the attached and free-living microbial communities

We performed a further network analysis to unravel the ecological role and patterns of co-occurrence for the microbes attached to *A. philoxeroides* and for the free-living microbes (both bacteria and fungi), respectively (Fig. 6). The ASVs with a relative abundance > 0.1% were used for network construction in this study. Network A is composed of 129 nodes and 382 edges (average degree of 5.922) and network F is composed of 147 nodes and 679 edges (average degree of 9.238) (Table 1). The high modularity of each co-occurrence network (A: 0.636, F: 0.508) suggests that each network has a modular structure (Newman 2006). Both networks have higher clustering coefficients compared with the random networks, suggesting that the networks have "small-world" properties (Watts and Strogatz 1998). In summary, Network A has less nodes, less correlations (edges) and a lower average degree than those of network F, but more modules were identified (Tables 1 and S2).

Of the two types of microorganisms, Network A has a lower ratio of positive links than Network F (A: 56.81% of all links, F: 74.67% of all links), which indicates less predominance of

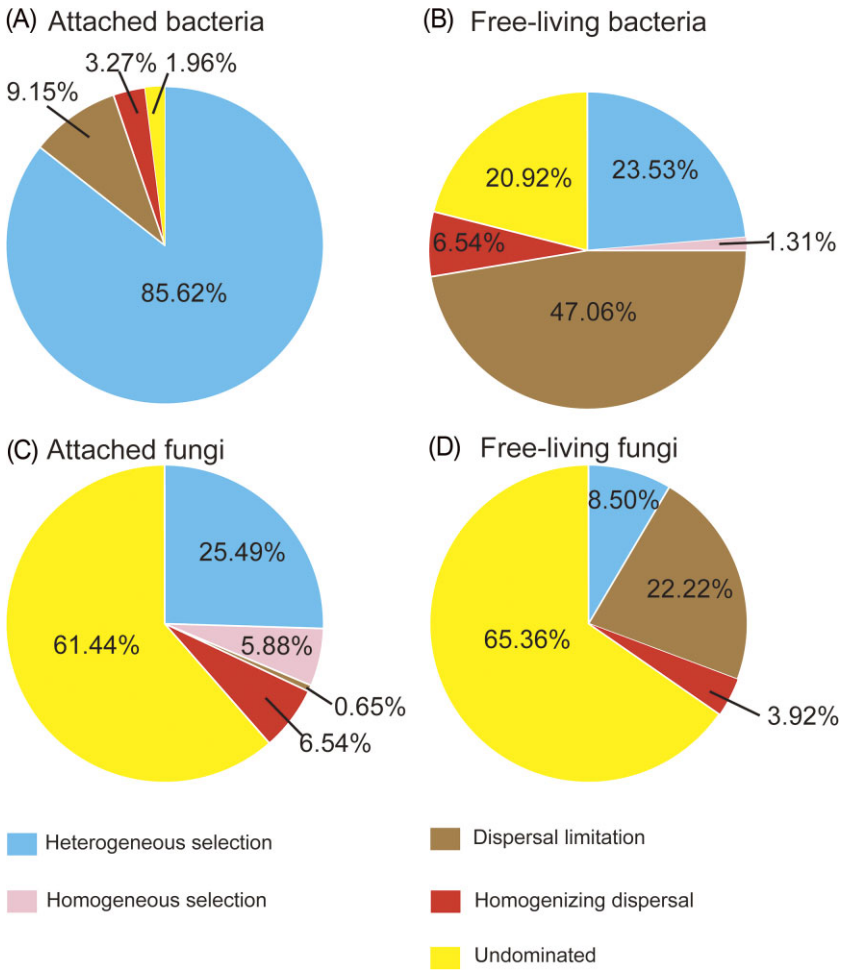

Figure 4. Relative importance of various ecological processes driving the assembly of microbial community across different sample groups. (**A**) Attached bacteria; (**B**) free-living bacteria; (**C**) attached fungi; (**D**) free-living fungi. Two null model-based β-diversity metrics (i.e. βNTI and RC_{Bray}) were applied to infer community assembly processes. Note that we calculated βNTI and RC_{Bray} separately for each combination of samples collected from the same habitat type and different times, and then calculated average β-diversity values from the dissimilarities for samples belonging to each pairwise combination of each group (**A**, **B**, **C** and **D**).

co-occurrence (Fig. 6; Table 2). More connections between bacteria and fungi were found in the attached microbe network (A: 19.63% vs F: 5.01%; Table 2) and also the proportion of negative links between bacteria and fungi is higher than that of negative links in the whole network (165/382), both intra- (33/61) and inter-modules (10/14). Over the two networks, the most frequent intra-phylum co-occurrence was found within the bacterial phyla *Actinomycetota*, *Alphaproteobacteria*, *Bacteroidota*, *Betaproteobacteria*, *Bacillota* and *Gammaproteobacteria*, while the most frequent intra-phylum co-occurrence was found within the fungal phyla *Basidiomycota* and *Chytridiomycota*. The most frequent inter-phylum co-occurrence was identified between *Actinomycetota*, *Alphaproteobacteria* and *Bacteroidota* with the other bacterial phyla. *Basidiomycota* is the most frequent fungal phylum that demonstrates inter-phylum co-occurrence with the other phyla (Tables 3 and S4).

Discussion

Processes controlling community assembly of the phyllospheric microorganisms

Although both deterministic and stochastic processes account for the variation in community composition (Zhou et al. 2014), the phylogenetic null model analysis in this study highlights that deterministic assembly processes play a more important role in governing attached microbes than free-living microbes. The previous studies point out that deterministic processes (e.g. environmental filtering and biotic interactions) were the primary ecological processes influencing the microbial community assembly during the initial successional phase of biofilm formation (Dini-Andreote et al. 2015). Leaf microtopography might regulate the assembly of attached bacterial communities through ecological niches or nutrient limitations (Yan et al. 2022). For example, leaf veins (especially veinlets) and the grooves between epidermal cells have been shown to facilitate the aggregation of microorganisms (Doan et al. 2020). Therefore, the characteristics of leaves might be essential in shaping the attached bacterial community.

Moreover, heterogeneous selection plays a much more vital role in the community assembly of the attached bacteria than of the attached fungi. Fungi showed potentially broader environmental thresholds for critical variables at the community level than bacteria, even if bacteria tended to be more metabolically flexible (Zhang et al. 2021b). The attached and the free-living microbes could exchange between the two communities (Grossart 2010). They were driven mainly by the ability of some microbes

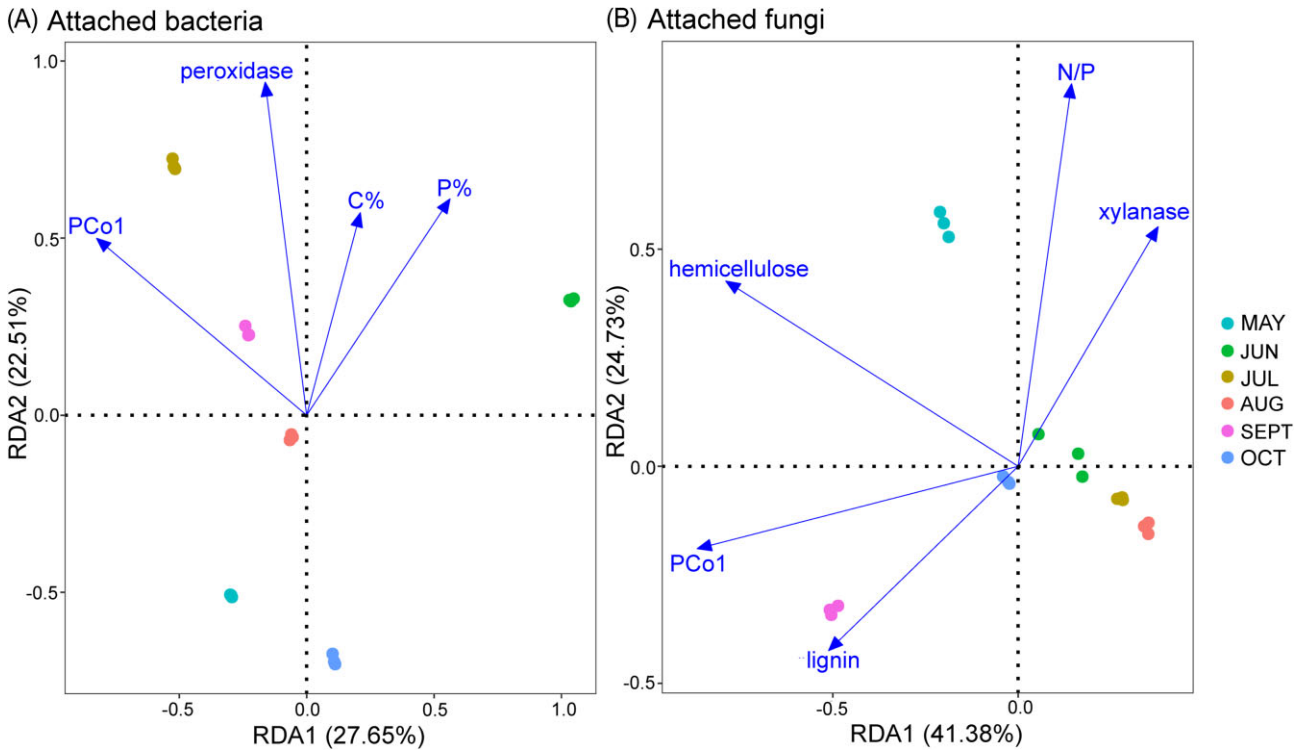

Figure 5. The significant environmental and physiological factors that correlated with the bacterial (**A**) and fungal (**B**) community composition attached to *Alternanthera philoxeroides* revealed by using an RDA. The values on axes 1 and 2 indicate the proportion explained by the combined significant factors. RDA, redundancy analysis.

to overcome the new environmental conditions imposed by disturbances (Lie´bana et al. 2019). We have also found that PCo1 of free-living microbes have significant correlations with seasonal assembly patterns of attached microbes. Nevertheless, the overlap of the attached and the free-living microbes along the growing season is relatively small (Fig. S1), which indicates that niche separation plays a more critical role in shaping bacterial communities (Leibold et al. 2004).

The community-level environmental thresholds have been considered a measure of niche breadth associated with specialized environmental variables (Jiao and Lu 2020, Zhang et al. 2021b). The attached bacterial community compositions were significantly correlated with the variations of leaves' C% and P%, as well as the activity of peroxidase, and the attached fungal community compositions were correlated with leaves' N/P ratio, the contents of hemicellulose and lignin, as well as significantly with the activity of xyalanase. Ecological community responses to environmental perturbations generally manifest as changes in species abundance and composition (McCune and Grace 2002).

The free-living microorganisms may have evolved to tolerate the environmental dynamics and thus are mainly shaped by dispersal limitation belonging to stochastic processes (Mujica-Alarcon et al. 2021, Liu et al. 2022). Our results reveal a higher effect of dispersal limitation on the free-living microbes from the surrounding water, especially free-living bacteria. This ecological process is considered to have resulted from a considerable spatial distance and/or steer environmental variance (Zhou and Ning 2017). The higher dispersal potentiality of free-living bacteria compared with free-living fungi is explainable because the smaller body and propagule sizes of bacteria might allow more accessible passive transport compared with fungi (Farjalla et al. 2012, Powell et al. 2015). Furthermore, we found a more significant influence of ecological stochasticity on the free-living bacterial community assembly than on the attached bacterial community assembly. This might be caused by a higher diversity of free-living bacteria than attached bacteria during the growing season (except for October, Fig. 2a). There is a similar study, using phylogenetic null modeling analysis, which found that the stochastic processes predominated in high-diversity communities, whereas the deterministic processes dominated in lower-diversity communities, associated with the reduction of specialized functions (Xun et al. 2019).

Co-occurrence/co-exclusion patterns of bacteria and fungi in the phyllosphere of *A. Philoxeroides*

Modules are connected network regions denselythat have more node connections inside the module than outside (Bissett et al. 2013). In this study, the higher modularity value of Network A may be associated with stronger niche differentiation in attached microbes than in free-living microbes (Chen et al. 2019). This suggests that the fungal and bacterial domains may be inclined to synergistically act or share similar ecological niches in the presence of macrophyte leaves, whereas they prefer to distribute into niches based on their own nutritional preferences and functional specificity on the surface of leaves (Chen et al. 2019).

On one hand, network A has more negative links than F, especially between bacteria and fungi within modules (Table 2), and

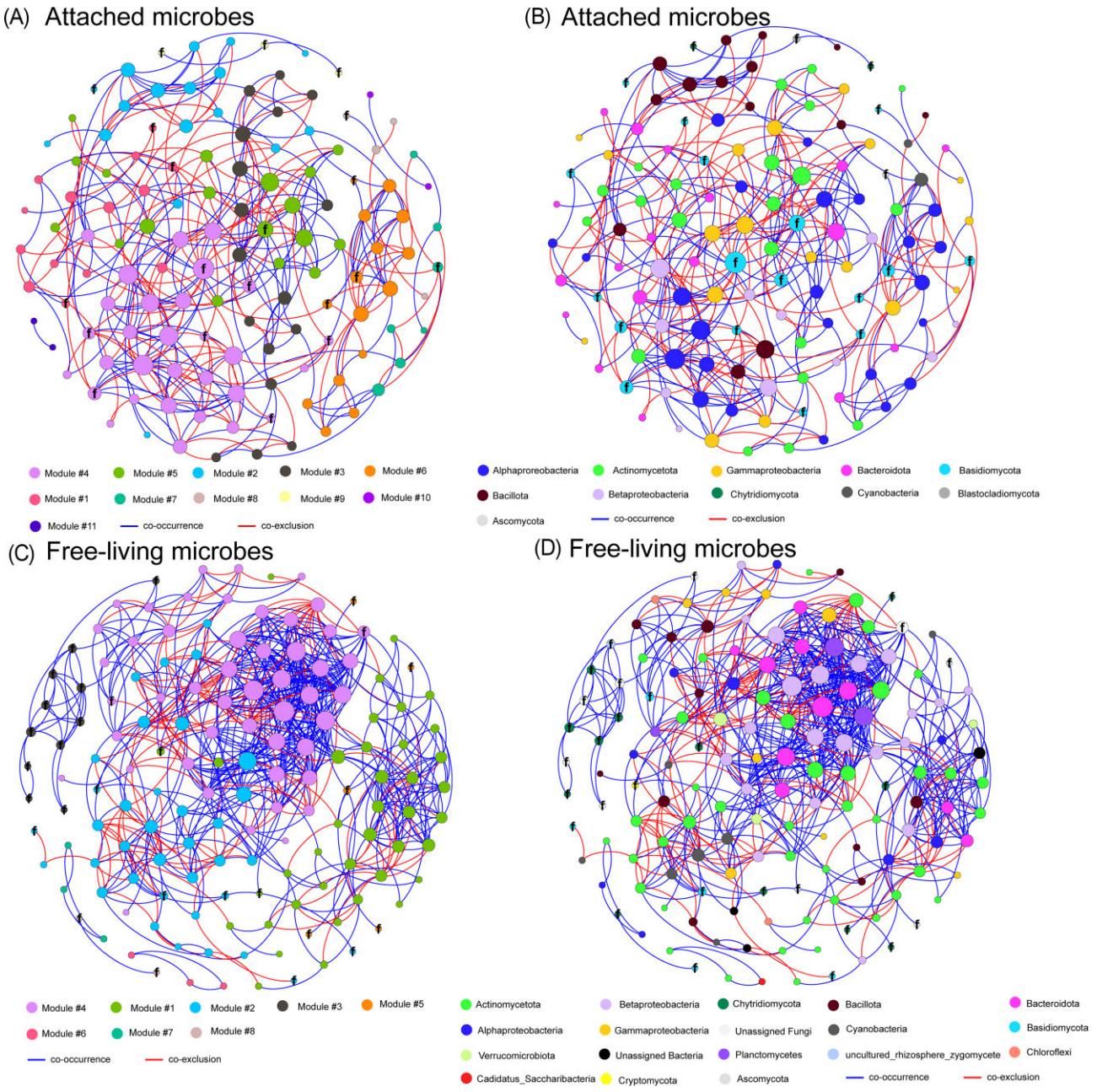

Figure 6. Networks of the attached (**A** and **B**) and free-living (**C** and **D**) microbial community (**A** and **C**: different modules are represented by different colors, **B** and **D**: different phyla are represented by different colors). Each node represents an ASV. A blue line indicates a positive correlation between the two individual nodes, whereas a red line indicates a negative correlation (according to the Spearman's correlation, $P < 0.05$, $R > 0.8$ or $R < -0.8$). The size of node indicates the degree. ASV, amplicon sequence variant.

these negative associations within communities can reinforce the stability of network structure (Mougi and Kondoh 2012). It indicates that microbes with different niche requirements may exhibit negative interactions (Barberán et al. 2012, Freilich et al. 2018), and the larger leaf vein density can supply more niches for microbes living on the leaf surface, which provides further support for the niche effect mechanism (Yan et al. 2022). There are also ecological relationships, such as competition or predation in the modules that contained higher negative links (Xu et al. 2018). There was a higher proportion of negative edges in the network of microbes attached to *A. philoxeroides* than in the free-living microbes, which suggests that competition or niche differentiation is more prevalent in the plant-associated microbiomes (Table 2). Co-exclusion patterns between fungi and bacteria have been found more frequently in the attached microbes (network A). The phylum *Basidiomycota* shows negative relationships with *Actinomycetota*, *Alphaproteobacteria*, *Bacteroidota*, *Betaproteobacteria*, *Bacillota* and *Gammaproteobacteria*. Negative correlations between different species could originate not only from direct competition but also from toxin production, environmental modification and differential niche adaptations (Faust et al. 2012). Otherwise, the different proportions of negative relationships suggest various

Table 1. Topological properties of the empirical species–species co-occurrence networks of microbes on the leaves of *Alternanthera philoxeroides* and in the ambient water.

		Attached microbes	Free-living microbes
Empirical network	Nodes	129	147
	Edges	382	679
	Module number	11	8
	Modularity	0.636	0.508
	Clustering coefficient	0.515	0.616
	Average path length	4.046	3.739
	Network diameter	9	9
	Average degree	5.922	9.238
	Graph density	0.046	0.063
Random network	Modularity (SD)	0.364 (0.011)	0.277 (0.008)
	Clustering coefficient (SD)	0.046 (0.009)	0.064 (0.006)
	Average path length (SD)	2.907 (0.018)	2.479 (0.006)
	Network diameter (SD)	5.569 (0.545)	4.135 (0.335)

The random networks were generated by rewiring all the links with the identical numbers of nodes and edges to the corresponding empirical network. The number in the parentheses indicate the standard deviation (SD) of the topological properties of 1000 random networks.

Table 2. Positive and negative links within/between the modules of two networks, and links between bacteria–bacteria, fungi–fungi and bacteria–fungi within/between the modules of two networks. The percentages are calculated by dividing the links by the sum of all the links in each network.

	Network A (attached)		Network F (free-living)	
	Links within modules	Links between modules	Links within modules	Links between modules
Total links	319	63	586	93
Positive links (%)	182 (47.64%)	35 (9.16%)	437 (64.36%)	70 (10.31%)
Negative links (%)	137 (35.86%)	28 (7.33%)	149 (21.94%)	23 (3.39%)
Bacteria–bacteria (positive/negative)	251 (148/103)	46 (29/17)	524 (380/144)	88 (66/22)
Percentage of bacteria–bacteria links in total links (positive%/negative%)	65.71% (38.74%/26.97%)	12.04% (7.59%/4.45%)	77.17% (55.96%/21.21%)	12.96% (9.72%/3.24%)
Fungi–fungi (positive/negative)	7 (6/1)	3 (2/1)	33 (33/0)	0
Percent of fungi–fungi links in total links (positive%/negative%)	1.83% (1.57%/0.26%)	0.79% (0.52%/0.26%)	4.86% (4.86%/0)	0
Bacteria–fungi (positive/negative)	61 (28/33)	14 (4/10)	29 (24/5)	5 (4/1)
Percentage of bacteria–fungi links in total links (positive%/negative%)	15.97% (7.33%/8.64%)	3.66% (1.05%/2.62%)	4.27% (3.53%/0.74%)	0.74% (0.59%/0.15%)

intensities of competition or niche differentiation in different environments (Ma et al. 2020).

On the other hand, we also detected a higher ratio of positive links between bacteria with fungi in the attached network than that of the free-living one (8.38% in network A vs 4.12% in network F, Table 2). This could result largely from microbe niche sharing, a common occurrence among bacterial networks (Shi et al. 2016). Network A has more intra-phylum linkages with a focus on *Actinomycetota*, *Alphaproteobacteria*, *Bacillota* and *Basidiomycota* than Network F (Tables 2 and S3). Strong ecological intra-phylum linkages are attributable to synergistic relationships, and the species from the same phylum tend to co-occur (Ju et al. 2014). The most abundant phylum in the attached fungal communities is *Basidiomycota*. Most of its ASVs are affiliated with *Tremellomycetes* and *Microbotryomycetes*, and this phylum is found more frequently in the later stages and network A. During the later stage of the growing season, *A. philoxeroides* is close to withering, and the addition of litter increased the proportion of *Basidiomycota*, including *Tremellomycetes* (Winder et al. 2013). On the contrary, the intra-phylum positive linkages of network F were almost present in each bacterial phylum, and the co-occurrence patterns of inter-phyla interactions are found in all microbial phyla, except for a few.

Table 3. Frequency of the observed co-occurrence (positive)/co-exclusion (negative) incidences between two taxa distributed intra-taxon/inter-taxon among bacterial and fungal phyla. The value was normalized by the number of total positive/negative links in a certain network, and values > 0.5% are shown here. The phyla that are affiliated with fungi are shown in bold font. Blank, does not exist.

			Network A (attached)		Network F (free-living)	
	Node1	Node2	Co-occurrence (%)	Co-exclusion (%)	Co-occurrence (%)	Co-exclusion (%)
Intra-taxon Interaction	Actinomycetota	Actinomycetota	6.02		4.42	3.98
	Alphaproteobacteria	Alphaproteobacteria	5.76	1.05	0.29	< 0.5
	Bacteroidota	Bacteroidota	1.31		2.36	
	Betaproteobacteria	Betaproteobacteria	1.05		6.48	
	Bacillota	Bacillota	5.76		1.18	
	Gammaproteobacteria	Gammaproteobacteria	1.83		0.74	
	Basidiomycota	**Basidiomycota**	1.31	0.52		
	Chytridiomycota	**Chytridiomycota**			1.33	
Inter-taxon interaction	Actinomycetota	Alphaproteobacteria	6.54	1.05	2.65	1.18
	Actinomycetota	Bacteroidota	1.31	2.36	4.71	2.65
	Actinomycetota	Betaproteobacteria	0.52	1.31	7.81	4.71
	Actinomycetota	Cyanobacteria	< 0.5		1.62	1.18
	Actinomycetota	Bacillota			1.62	1.03
	Actinomycetota	Gammaproteobacteria	< 0.5	7.33	0.88	< 0.5
	Actinomycetota	Planctomycetes			1.77	0.59
	Actinomycetota	Unassigned Bacteria			0.59	< 0.5
	Actinomycetota	Verrucomicrobiota			1.18	< 0.5
	Alphaproteobacteria	Bacteroidota	2.88		< 0.5	1.18
	Alphaproteobacteria	Betaproteobacteria	1.31	3.66	0.74	0.88
	Alphaproteobacteria	Cyanobacteria	0.79	0.26		< 0.5
	Alphaproteobacteria	Bacillota	< 0.5	2.09	0.59	< 0.5
	Alphaproteobacteria	Gammaproteobacteria	1.83	4.71		< 0.5
	Bacteroidota	Alphaproteobacteria	< 0.5	0.79		
	Bacteroidota	Betaproteobacteria	1.31	0.52	9.13	< 0.5
	Bacteroidota	Bacillota	< 0.5	3.93	< 0.5	
	Bacteroidota	Gammaproteobacteria	2.36	0.52	1.18	
	Bacteroidota	Verrucomicrobiota			0.88	
	Betaproteobacteria	Cyanobacteria			< 0.5	0.74
	Betaproteobacteria	Bacillota	0.79	< 0.5	0.44	< 0.5
	Betaproteobacteria	Gammaproteobacteria	2.88	0.52	1.33	< 0.5
	Betaproteobacteria	Planctomycetes			2.95	< 0.5
	Cyanobacteria	Gammaproteobacteria		0.52	< 0.5	
	Bacillota	Gammaproteobacteria	0.52		0.88	1.03
	Bacillota	Planctomycetes			0.59	
	Planctomycetes	Bacteroidota			1.91	
	Verrucomicrobiota	Betaproteobacteria			0.74	< 0.5
	Basidiomycota	Actinomycetota	1.57	0.52	0.59	
	Basidiomycota	Alphaproteobacteria	3.66	2.36		
	Basidiomycota	Bacteroidota	1.05	2.09		
	Basidiomycota	Betaproteobacteria	0.52	1.83		< 0.5
	Basidiomycota	Cyanobacteria	0.52		< 0.5	< 0.5
	Basidiomycota	Bacillota	0.79	1.31	0.44	< 0.5
	Basidiomycota	Gammaproteobacteria	< 0.5	3.40		
	Blastocladiomycota	**Chytridiomycota**	0.52			
	Chytridiomycota	**Unassigned Fungi**			0.74	
	Unassigned Fungi	Betaproteobacteria			0.59	
	Unassigned Fungi	**Chytridiomycota**			1.33	

Conclusions

In this study, we investigated the diversity, community composition and co-occurrence pattern of microbes attached to *A. philoxeroides* and the surrounding free-living microbes as well. We found that the alpha diversities of the attached and free-living microbial community were influenced by the time of plant growth, but there was no significant difference in the alpha diversity between the attached and free-living microbes. Both the bacterial and fungal community compositions showed notable differences between the attached and free-living habitats. Deterministic processes play a much stronger role in the community assembly of the attached bacteria, while ecological stochasticity made a larger contribution to the assembly of attached fungi communities. The attached microbial network was structurally simple but highly modular compared with the free-living microbial network. There are more intra-phylum links in the attached microbes and distinct co-exclusion patterns between bacteria and fungi in the modules,

which indicates the presence of specific bacterial and fungal interactions in the phyllosphere. Generally, the study will be helpful in understandingthe microbes and their interactions in the phyllosphere of *A. philoxeroides*, an key invasive species under national management and control .

Supplementary data

Supplementary data are available at FEMSEC online.

Author contributions

Biying Zhao: research design, experiment performance, data analysis and paper writing; Jiangjun Chen and Zhicong Dai: data collection and analysis; Yujuan Zou: data curation and visualization; Peng Xing and Qinglong L. Wu: research design, funding acquisition and paper revision.

Conflicts of interest. The authors declare that the research was conducted in the absence of any conflict of interest.

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