Disease-induced Changes in Plant Microbiome Assembly and Functional Adaptation

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Research

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Abstract

Background: Plant microbiome is an integral part of the host influencing its growth and health. The increasing evidence indicates that plant rhizosphere may recruit beneficial microbes to suppress soil-borne pathogen, but the ecological mechanisms that govern plant microbiome assembly and functions under disease in both below and aboveground compartments are not fully understood. Here we examined both bacterial and fungal communities from soils (rhizosphere and bulk soil) and multiple plant compartments (e.g. root, stem, and fruit) of chili pepper (*Capsicum annuum* L.) at two pepper production sites, and explored how *Fusarium* wilt disease (FWD) affect the assembly, co-occurrence patterns, and ecological functions of plant-associated microbiomes.

Results: Our data demonstrated that FWD had less impact on reproductive organ (fruit) than on vegetative organs (root and stem), with the strongest impact in the stem upper epidermis. Fungal intra-kingdom networks presented lower stabilities and their communities were more sensitive to FWD than the bacterial communities. Moreover, the diseased pepper was more susceptible to colonization by other pathogenic fungi, but they may recruit potential beneficial bacteria to facilitate host or offspring survival, and FWD may enhance the ecological importance of fungal taxa in the interkingdom network. Further, metagenomic analysis revealed that several potential protective functional genes encoding detoxify and biofilm formation were significantly enriched in the diseased pepper.

Conclusion: Together, these results significantly advance our understanding of pepper microbiome assembly and functions under biotic stress. Our work highlights the diseased plant and the aboveground compartments harbor a potential of beneficial microbiomes and functions that, in concert, can provide potential critical data for harnessing the plant microbiome for sustainable agriculture.

Background

Plants and their associated microbiota co-evolve for millions of years and form a ‘holobiont” that provides essential functions in host phenotypes and fitness [1–5], including nutrient acquisition [6–8], abiotic stress tolerance [9] and disease suppression [10, 11]. In consequence, manipulating the plant microbiome is increasingly considered as an environmentally sustainable way to promote plant protection and agriculture production. Uncovering the fundamental ecological patterns that govern plant microbiome assembly, plant-microbe interactions, and functions are the prerequisites for engineering plant microbiomes. Previous studies have suggested that plant microbiome assembly is shaped by multiple biotic and abiotic factors, such as host selection caused by plant compartment and host genetics [12–14], climate, and soil type [15, 16]. Apart from host selection and herbivorous insects [17], pathogen invasion [18, 19] is probably the most influential biotic stress for plant microbiome assembly. Probing microbiome assembly along the soil-plant continuum under pathogen invasion will pave the way for harnessing the plant microbiome to enhance plant health and maximize crop production.

A growing body of experimental and observational literature has been providing evidence that rhizosphere is a critical zone of plant [9, 13, 20–22]. Root exudates and other root-derived molecules are believed to play an important role in altering rhizosphere microbiome [23–26]. On the other hand, phyllosphere, which refers to the aerial parts of plants, may play essential, but often overlooked roles on plant productivity and ecosystem function [27–29]. Studies have suggested that phyllosphere microbiome may provide numerous benefits to plants, including enhancement of stress tolerance [30], promotion of growth and reproduction [31], and protection from foliar pathogens [32]. Additionally, several recent studies provided evidence that aboveground pathogen or insect infections will adjust their rhizosphere communities [24, 25]. Belowground rhizosphere microbiome also plays a key role in determining aboveground productivity and health [33, 34]. Bai et al. established leaf- and root-derived microbiota culture collections in *Arabidopsis thaliana*, and found an extensive taxonomic overlap between them [35]. These studies indicated that plant below and aboveground microbiomes are systematically linked. Previous studies have focused on the below and aboveground microbiome separately and independently, and our understanding of the combined below and aboveground compartments as a whole is still limited.

Different components of plant microbial communities (bacteria, fungi, protist, archaea, and viruses, etc.) respond to the external stress differently [16, 36, 37]. For example, soil bacterial networks are less stable under drought than fungal networks [16]. However, few studies have assessed the response of microbial communities to plant disease, at different plant compartments (below and aboveground) as well as different microbial components (bacterial and fungal). Also, our understanding of the functional group changes is limited under plant disease. In this investigation, we employed chili pepper (*Capsicum annuum* L.), an economically important Solanaceae plant in China, and *Fusarium* wilt disease (FWD) to investigate the questions on the responses of microbial communities to plant pathogen invasion.

FWD is caused by *Fusarium oxysporum*, the most common species in *Fusarium*. This pathogen is a classical soil-borne pathogen that attacks a wide variety of economically important crops [38–40], including banana [41, 42], watermelon, and solanaceae plant of tomato, eggplant and chilli pepper. The pathogens enter through the roots and interfere with the water conducting vessels of the plant causing the brown vascular bundle and wilt symptoms. Chilli pepper is one of the major agricultural crops in China, with over 10^6 ha of total cultivated area, and accounts for about 40% of the world's pepper cultivating area, ranking first in the world. FWD caused by *Fusarium oxysporum* f.sp. *capsici* in pepper [43], leads to significant yield losses annually.

Plants consist of different organs, which are classified as vegetative (roots, stems, and leaves) and reproductive (fruits, flowers, and seeds), and each of them is responsible for certain functions. Given that plant may enhance offspring fitness [25, 44], we hypothesized that the disease occurrence would have greater impact on vegetative organ than in reproductive organ, and the infected plant may recruit protective microbes. Since fungal communities have been found to be more responsive than bacterial communities to vegetation change [36], and fungi are the first consumers of belowground inputs of plant derived carbon [45–47], we expected fungal communities are more sensitive to FWD. Given much

evidence linking the taxonomic composition and ecological functions [27, 48–50], we also hypothesized the disease-induced changes in taxonomic composition influence their functional adaptation. In this study, we investigated the taxonomic and functional differences between healthy and diseased pepper associated microbiomes using both amplicon sequencing (profiling bacterial and fungal groups) and metagenomic sequencing in Guizhou, China, where pepper is a significant crop and the incidence of FWD is high. The microbiome assembly and co-occurrence network, as well as the functional genes were used to identify and to evaluate the microorganisms associated with disease invasion.

Materials And Methods

Sampling

All samples were collected from the main pepper production fields in Huishui (25°48'41"N, 106°31'24"E) and Guiyang (26°29'31"N, 106°39'16"E92.1 km apart), in Guizhou province, southwest China. Two sites are located in subtropical monsoon climate zone, with the same annual mean temperature of 15.8°C, and annual mean precipitation of 1213.4 and 1259.8 mm respectively. Pepper plants at maturity stage were sampled in August 2018. In each site, pepper plants showed no wilt symptoms and were negative for pathogen isolation were classified as healthy plants, while those showed wilt, brown vascular bundles symptoms and were positive for pathogen isolation (confirmed by morphological and molecular data) were classified as diseased plants (Fig S1). From three adjacent plots of each site, healthy and diseased plants with three replicates were collected respectively, and each replicate consisted of a composite sample by mixing three individual pepper plants. The rhizosphere soil and corresponding bulk soil of each individual and the plant samples were collected and transported on dry ice to the laboratory and frozen at -80℃.

DNA extraction and amplification sequencing

Root and fruit samples were fractionated into episphere and endosphere compartments, representing microbes residing on the root and fruit surface or inside, respectively. For episphere microbial DNA extraction, 10-20 g fruits or 3-5 g roots were put in sterile bottle or polystyrene tubes containing release buffer (0.1 M Potassium Phosphate, 0.1% Glycerol, 0.15% Tween 80, pH 7.0; 150 ml for fruit and 35 ml for root), and subjected to sonication at 40 kHz for 1 min and then washed for 4 min at 200 rpm on a shaker [14]. This procedure was repeated twice, and the washes were then filtered through a 0.22 μm nitrocellulose membrane filter (BOJIN, Germany). These filters containing episphere microorganisms were frozen on -80℃ for further processing.

For endosphere microbial DNA extraction, about 5 g of fruits or roots were treated as above to dislodge episphere. After that, the fruits or roots were rinsed with 70% ethanol for 5 min, followed by 5.25% sodium hypochlorite solution for 5 min, and 70% ethanol for 30 sec, finally washed with sterile H_2O for 5 times for surface sterilization. The treated fruits and roots samples were ground using sterile mortars and frozen

on -80℃ for further processing. The pepper stem samples were divided into stem upper, stem middle, and stem bottom, and each part was divided into epidermis and xylem, respectively (Fig S2). These microbe-enriched epidermis and xylem fractions were ground and transferred to 2 ml tubes. Totally, we divided each plant sample into 12 compartments, including the bulk soil (BS), rhizosphere soil (RHS), root episphere (Repi) and endosphere (Rendo), stem bottom epidermis (SB-epidermis) and xylem (SB-xylem), stem middle epidermis (SM-epidermis) and xylem (SM-xylem), stem upper epidermis (SU-epidermis) and xylem (SU-xylem), fruit episphere (Fepi) and endophere (Fendo) (Fig. 1a and Fig S2).

Total DNA was extracted from the aforementioned samples using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, USA) following manufacturer's instructions. The V5-V6 region of bacterial 16S rRNA gene (799F-1115R) [14, 51], and the ITS2 region (fITS7-ITS4) of fungal ITS [16, 52, 53] were amplified (Table S1). The primers sequence and PCR amplifications were shown in Table S1. The library was loaded onto an Illumina HiSeq 2500 platform using the PE250 sequencing at the MEGIGENE Biological Company (Guangdong, China).

Amplicon sequencing data analyses

The 16S rRNA gene and ITS sequences were processed using USEARCH v.10.0 [54] and Quantitative Insight into Microbial Ecology (QIIME1.9.1) [55]. In brief, primer sequences and low-quality reads ends below Q30 were trimmed. Paired 16S rRNA and ITS amplicon sequencing reads were merged to a single sequence, and the ITS paired reads were trimmed to 200 bp and then quality-filtered (maximum expected error 0.5) in USEARCH. All correct biological reads were picked at 100% sequence similarity using unoise3 [56] command with default parameters in USEARCH. Taxonomic assignment for bacteria was performed using SILVA reference database (Version 12_8) [57], and that for fungi was done using UNITE database (v7.0) [58]. Bacterial zero-radius operational taxonomic units (ZOTUs) assigned to chloroplast, mitochondria, or viridiplantae, as well as fungal ZOTUs assigned to plant or protist were removed. ZOTUs represented by less than 2 sequences were also removed to avoid possible biases. MetagenomeSeq's cumulative sum scaling (CSS) was used as a normalization method for bacterial and fungal beta-diversity analyses [59]. Both alpha-diversity and beta-diversity of bacterial and fungal microbiome were calculated in QIIME 1.91, and the bacterial and fungal ZOTU table was rarefied to 10250 and 5005 reads for alpha diversity estimates, respectively. Fungal ZOTUs were assigned into functional guilds using the online application FUNGuild (http://www.stbates.org/guilds/app.php) [60], and the confidence ranking of "Highly Probable" and "Probable" were kept to reach the high accuracy.

Metagenomic sequencing workflow and data analyses

Based on the amplification sequencing results, we selected the pepper samples of stem upper epidermis and root endosphere in the Huishui site for metagenomic sequencing. Twelve DNA samples were sent to the Shanghai Majorbio Bio-pharm technology for sequencing on an Illumina NovaSeq 6000 instrument (150-bp paired-end reads). We get approximately 20GB clean data for each DNA sample. To remove host-

derived sequences, we used Bowtie2 2.4.1 [61] to build host genome database (*Capsicum annuum* cultivar Zunla-1, GenBank: ASJU00000000.1), and the metagenomic data were mapped against the host genome database. The unmapped fraction was quality trimmed and filtered. Afterward, genes were predicted over contigs by using Prokka [62]. All predicted genes were combined and clustered using cd-hit into a non-redundant gene set by setting the similarity threshold as 95%. Functional annotation was performed by eggnog-mapper 0.13.1 [63] using diamond comparison to eggNOG databases [64]. We reorganized the annotations result to KEGG Orthology (KO) profiles with summing up the abundance of genes affiliated to the same KO [65], Clusters of Orthologous Groups of proteins (COG) functional categories [66], antibiotic resistance genes with ResFams [67], and CAZymes [68]. Functional diversity was calculated using QIIME 1.91, and the significance of FWD on functional dissimilarity was tested with betadisper function in the vegan package in R [69]. The different function exploration was conducted by LEfSe analyses (Galaxy web application: http://huttenhower.sph.harvard.edu/galaxy/) [70].

Statistical analyses

Alpha diversity indices (Shannon index, Chao1 index, and number of observed species) were calculated using QIIME 1.91 (alpha_diversity.py). The significance of differences between samples from healthy and FWD in each host compartment was tested using the Wilcoxon rank-sum tests (Wilcoxon rank.tests in R). The linear mixed model (LMM) was employed to identify the major drivers of alpha diversity and composition, and the strength of the variables was compared by a type II ANOVA test and marginal pseudo-R^2 was calculated for the model [71]. Bray-Curtis distance matrices were calculated and visualized using non-metric multi-dimensional scaling (NMDS) ordinations to assessed bacterial and fungal beta-diversity using QIIME 1.91. The significance of different factors on community dissimilarity was tested with permutational multi-variate analysis of variance (PERMANOVA; *Adonis* function from vegan package, in R) [72] analyses based on 1999 permutations, using the Bray-Curtis distance matrix as an input. The PERMANOVA analysis was also calculated to test the dimensions of FWD and sampling site in single compartments. To calculate beta dispersion, we used the betadisper function in the vegan package in R, which is a multivariate analogue of Levene's test for homogeneity of variances. Differential ZOTUs between healthy and diseased plant within each host compartment were calculated by differential abundance analysis.

Co-occurrence network analyses

The co-occurrence patterns were constructed by calculating multiple abundance correlations and similarities based on the genus level matrices with co-occurrence network (CoNet) app, using Cytoscape [73]. We considered a co-occurrence to be robust if the Spearman's correlation coefficient (ρ) > 0.70 and significant ($P < 0.05$). The P values were merged using Brown's method and then adjusted using the Benjamini-Hochberg procedure to reduce the chances of obtaining false-positive results [74]. The networks were visualized with the interactive platform Gephi [75]. Nodes represented individual microbial

genus in the microbiome network. Network edges represented the pairwise correlations between nodes, suggesting the biologically or biochemically meaningful interactions.

The calculated topological characteristics of the bacterial and fungal networks included the numbers of co-occurrence (positive) and mutual exclusion (negative) interaction type, average path length, network diameter, average clustering coefficient, average connectivity, and modularity (Table S8). The roles of individual nodes were estimated by three-node parameters: degree, the number of direct correlations to a node in the network; closeness centrality, which quantified to what extent a node connected to different modules; and betweenness centrality, which quantified how well the node connected to different modules [12].

Results

FWD affected pepper microbiome assembly

In total, 8,672,206 bacterial 16S and 7,677,988 fungal ITS high-quality reads from 144 samples were obtained, and these reads were sorted into 14,976 bacterial and 4,277 fungal ZOTUs. To examine the dimensions in which the multiple factors shape the pepper microbiome, we assessed the relative contribution of multiple factors in terms of plant compartment, *Fusarium* wilt disease (FWD), and sampling site in shaping the microbial communities. NMDS ordinations and PERMANOVA analysis showed that the greatest effect on the total microbiome was exerted by the compartment (R^2 = 0.47 for bacteria and R^2 = 0.53 for fungi), followed by FWD (R^2 = 0.06 for bacteria and R^2= 0.03 for fungi), and lastly by sampling site (R^2= 0.01 for bacteria and R^2= 0.02 for fungi) (Fig.1b and Table S2). FWD explained higher variation of fungal community than of the bacterial in compartments of root endosphere, stem bottom epidermis, stem middle epidermis and xylem, stem upper epidermis and xylem, and fruit episphere (Fig. 1c, Table S3, and Table S4). Notably, fungal community was less affected by FWD in pepper fruit than that in stem and root (root/stem/fruit R^2: 0.17/0.22/0.15, in average, respectively; Fig. 1c and Table S4). For stem, FWD effect on fungal community was stronger in epidermis than that in xylem (epidermis/xylem, R^2: 0.24/0.13 in stem bottom, 0.23/0.14 in stem middle, 0.39/0.16 in stem upper, respectively; Fig. 1c, Fig S4, and Table S4). Through all compartments, the greatest effects of FWD on fungal community were found in stem upper epidermis and root endosphere (R^2 = 0.39, P = 0.001 in stem upper epidermis, and R^2 = 0.25, P = 0.001 in root endosphere) (Fig. 1c, and Table S4). In contrast, sampling site explained higher variation of bacterial communities than FWD in most compartments (Fig. 1c, Fig S3, and Table S3). In addition, both bacterial (P = 0.012) and fungal communities (P = 0.037) in diseased plant were more variable than the healthy based on beta-dispersion using Bray-Curtis dissimilarity (Fig. 1d). In the bacterial community, the diseased plant were more variable than the healthy in bulk soil, root endosphere, stem bottom epidermis and xylem, stem upper epidermis, and fruit episphere (Table S6). In the fungal community, the diseased plant were more variable than the healthy in bulk soil, rhizosphere soil, stem middle xylem, stem upper epidermis, and fruit episphere (Table S6).

We used linear mixed models (LMMs) to explore the most important driver of microbial alpha diversity, and the result showed that both bacterial and fungal Shannon diversity were mainly influenced by compartment ($P < 0.0001$, Table S5). FWD had stronger effect on fungal Shannon diversity ($P = 0.00172$) than on bacterial ($P = 0.023$, Table S5). Remarkably, fungal Shannon diversity significantly decreased in stem upper epidermis (30%), stem bottom epidermis (28%), root endosphere (28%), and rhizosphere soil (19%) under FWD ($P < 0.05$, Fig. 1f). In contrast, sampling site had stronger effects on bacterial Shannon diversity ($P = 0.006$) than on fungal community ($P = 0.831$, Table S5).

The LMMs of compositional variations showed that FWD had significant effects on the relative abundance of fungal class *Tremellomycetes* ($P < 0.05$) but not on any bacterial phyla (Table S7, Fig S5h, and Fig S6a). The differential abundance analysis showed a higher relative abundance of class *Tremellomycetes* in the diseased plant (Fig. 2a), and this class belonged to the same fungus functional guild Saprotroph (Yeast) (Fig S6b). The relative abundance of several pathogenic fungi in the genera *Diaporthe*, *Fusarium*, *Gibberella*, *Phomopsis*, *Plectosphaerella*, *Stemphylium*, and *Cryptococcus* were also significantly higher in diseased plant root and stem ($P < 0.001$), but not in fruit (Fig. 2a&c). However, several potential beneficial taxa in the genera *Pseudomonas*, *Streptomyces*, *Klebsiella*, *Enterobacter*, *Microbacterium*, *Bacillus*, *Chitinophaga*, and *Citrobacter* were significantly enriched in the diseased plant ($P < 0.001$, Fig. 2b&d and Fig S6c).

FWD affected pepper microbiome co-occurrence network

To further explore how FWD effects on pepper microbiome co-occurrence patterns, we conducted bacterial-bacterial, fungal-fungal intra-kingdom networks, and bacterial-fungal interkingdom networks. The intra-kingdom co-occurrence networks analyses showed that the bacterial networks had properties that suggest higher stability than fungal networks. A higher number of nodes and edges were recorded in bacterial networks than fungal networks (Fig. 3 and Table S8). Further, the edges of top 10 hub nodes that had higher degree and centrality values (closeness) in bacterial networks were primarily negative particular for the healthy network (Fig. 3b&c). In contrast, most edges of fungal networks were primarily positive (Fig. 3b&c). Moreover, the healthy bacterial network showed higher complexity (based on the number of nodes and edges) than the diseased network, with the contrasting pattern in the fungal network (Fig. 3b, d&e and Table S8).

The interkingdom co-occurrence networks indicated a more important role of fungal taxa played in diseased network. The number of nodes and connections of fungal taxa increased in diseased network compared with the healthy one, while an opposite pattern was observed in bacterial taxa (Fig. 4a-c and Table S8). These increased connections of fungal taxa were mainly related to bacterial-fungal (BF) and fungal-fungal (FF, especially the positive one) correlations, and the decreased edges of bacterial taxa were related to the bacterial-bacterial (BB) correlations (Fig. 4d). Additionally, the interkingdom correlations between bacterial and fungal taxa (BF) were primarily negative (92.1% in healthy and 78.3% in diseased), whereas positive correlations dominate intrakingdom correlations (60% BB and 98% FF in

healthy, and 66% BB and 99% FF in diseased; Fig. 4d). The top 10 hub species were wholly belonged to bacterial taxa in the healthy network, while fungal taxa accounted for a half number in the diseased network (Fig. 4e&f, Table S10). The similar patterns were also revealed in most single compartment networks (Fig S7). Overall, the intra- and interkingdom networks analyses indicated higher stability properties in the bacterial networks than the fungal networks, and FWD decreased the complexity of bacterial taxa, whereas increased the complexity of fungal taxa.

FWD affected pepper microbiome functions

Metagenomic sequencing was conducted to explore the impact of FWD on the functional genes in pepper microbiome; two compartments (stem upper epidermis and root endosphere) having high variations of microbiome assembly between healthy and diseased pepper were selected for this analyses.

The results indicated significant effects of FWD on KO, CAZ, and ResFam functional profiles in stem upper epidermis microbiome ($P < 0.05$, Fig.5a), but FWD had no significant effects in root endosphere microbiome in any functional profiles ($P > 0.05$, Fig S8a). FWD significantly decreased the functional diversity of KO ($P = 0.0314$), COG ($P = 0.0074$), and Resfam ($P = 0.0065$) profiles in stem upper epidermis microbiome, but showed no significant effects in root endosphere microbiome ($P > 0.05$, Fig. 5b).

We performed differential abundance analysis to identify how FWD affected the functional properties. The results demonstrated that the microbiome in stem upper epidermis possessed higher number of specific functional genes than in root endosphere (Table S11). The *phoD* Alkaline Phosphatase Gene (K01113) and mprF:peptide antibiotic resistance gene was significantly enriched in healthy root endosphere, and vancomycin resistance gene clusters were significantly enriched in healthy stem upper epidermis ($P < 0.05$, Fig. 5c, Fig S8c&f, and Table S12). Notably, in diseased plant, functions of *csgD* LuxR family transcriptional regulator (K04333) was significantly enriched in root endosphere, as well as UDP-glucuronosyltransferase (GT1) and replication, recombination and repair (COG_L) were significantly enriched stem upper epidermis ($P < 0.05$, Fig. 5c, Fig S8c-e, and Table S12).

Discussion

FWD had less impact on reproductive organ than on vegetative organs

Overall, the fungal community was less affected by FWD in fruit than that in stem and root. The change of fungal community was associated with co-infections of other fungal pathogens in root and stem, but not in fruit. Thus, the reduced FWD effect on fruit than root and stem may represent a life-history tradeoff strategy of the plant to survive the next generation (fruit and seed) rather than investing the contemporary diseased individuals. The secondary metabolites such as capsaicinoids produced by pepper may inhibit the growth of certain fungi [76]. For stem, the FWD impact on bacterial and fungal community was

stronger in epidermis than that in xylem. Compared to xylem, epidermis is a more favorable niche for microbes in terms of accessible organic nutrients (such as small sugars) [77, 78]. For root, a stronger FWD effect on bacterial and fungal community was detected in the endosphere, where epidermis and xylem were not distinguished in this study.

The microbial communities in diseased plants were more variable than the healthy one in most compartments by comparing the community dissimilarity. This is contrary to the expectation that, based on homogeneous selection [79], the same environment selection pressure leading to more similar structures among communities. The microbial communities associated with hosts are probably shaped by a wide variety of environmental and host-related factors. A recent study has found that host selection (i.e. compartment niche and species) has greater determining effect over environmental factors in shaping the plant microbiome assembly [14]. The strong effect of the host compartment seen in our pepper data (0.47 for bacteria and 0.53 for fungi) was also seen in studies of sorghum (42.1%) [80] and *Populus* [15, 81] of fungal community, and maize (47.5%)-wheat/barley (58%) [14] of bacterial community. This study could provide additional evidences for niche occupation theory of plant microbiome assembly [81, 82], under both healthy and diseased conditions. Having observed a predominant effect of host compartment on microbial community composition, we propose that the disease invasion may lessen the plant effect and, thereby, leading to a higher community dissimilarity in diseased plant.

Fungal communities are more sensitive to FWD than bacterial communities

FWD explained higher variation of fungal community than the bacterial in most compartments. The co-occurrence networks analyses indicated that the network stability of bacterial was higher than that of fungal, a possible explanation on their different responses to FWD. The co-occurrence interactions has revealed as a powerful tool to unravel the role of the microbiota in response to biotic stress [19]. The mutually negative interactions, indicating ecological competitions, could improve microbiome stability by dampening the destabilizing effects of cooperation [83]. Hosts may benefit from microbial competition that result in better resistance to external stresses, as different taxa can complement each other [48]. In this study, bacterial networks and their hub species in both healthy and diseased plants revealed a higher number of negative correlations than those of fungal networks, suggesting that bacterial networks may present higher stability than fungal networks. The bacterial communities associated with pepper were, overall, highly affected by sampling site than the fungal communities. Bacteria and fungi have fine differences in body size, metabolic activity and dispersal potential [84-86], which may exert influences on the relative importance of species sorting and dispersal limitation.

Microbiomes in different compartments responded differently to FWD, and the strongest response from the stem upper epidermis microbiome. One possible explanation is that disease-induced changes in plant physiological characteristics, such as water relations [87] affect the aboveground parts of a plant, for

instance, the moisture content of stem upper epidermis decreased by 72.4% in diseased plant (Fig S8b). The initial symptom of FWD is root necrosis [39, 40], and fungi are the primary consumers of belowground inputs of plant derived carbon [45-47], which may explain why the mycobiome in the root endosphere responded strongly to FWD.

Our data showed that FWD decreased the complexity of bacterial networks but increased the fungal networks in pepper microbiomes. The contrasting responses of bacterial and fungal networks parallel recent observation based on soil macroecological patterns of *Fusarium* wilt [88]. Our data showed a higher complexity in diseased fungal intra-kingdom network, and a higher degree of fungal taxa in diseased interkingdom network, compared with the healthy one. The increasing connections of fungal taxa may be associated with the crucial role of fungi played in litter decomposition [89]. In addition, the result of increased positive fungal-fungal (FF) connections in diseased network suggests that, FWD may promote the destabilizing properties due to the positive intra-kingdom correlations. Bacterial and fungal groups were reported to compete for plant derived substrates [47], which could explain that positive correlations dominate within each kingdom, while negative correlations dominate between bacteria and fungi (Fig. 4d).

Disease-induced changes of microbiome taxonomic compositions and functions

Our study indicated that several bacterial taxa with potential pathogen-suppressing effects were enriched in compartments of diseased plant, significantly ($P < 0.001$). These potential pathogen-suppressing bacteria including the genera *Pseudomonas* [42, 49], *Streptomyces* [90], *Klebsiella* [41], *Enterobacter* [41, 91], *Microbacterium* [24], *Bacillus* [42, 49, 92], *Chitinophaga* [11], and *Citrobacter* [93].

Previous work has suggested that bacterial microbiota is essential for protection against pathogenic fungi and oomycetes [82]. Our finding provided evidence on the critical role of bacterial taxa and the plants involved in the “cry for help” strategy, of which, plants actively involve their microbial partners to maximize their survival and growth when affected by external stress, and is likely a survival strategy conserved across the plant kingdom [17, 20, 94]. Several recent studies have provided evidence of this strategy that aboveground pathogen infection will induce the assemblage of a plant-beneficial bacterial consortium in root microbiome [24, 25]. Based on our result, we propose the possibility of soil-borne pathogen infection (e.g., *Fusarium* wilt disease) could also drive the recruitment of beneficial microbes in aboveground parts. Intriguingly and recently, Liu et al. have provided evidence on the recruitment of beneficial microbes in wheat rhizosphere and root endosphere to suppress soil-borne pathogen (*Fusarium pseudograminearum*), and they showed that the beneficial microbe *Stenotrophomonas rhizophila* can act as an early warning agent for plant defense and boost plant defense in the aboveground parts (when the pathogen was present) [44].

Apart from the microbial assembly data, metagenomic data also showed that the microbiomes in the diseased pepper plant were associated with higher number of functional genes encoding detoxify and biofilm formation. UDP-glucuronosyltransferases (GT1) and CsgD LuxR family transcriptional regulator (K04333) were significantly enriched in the diseased pepper stem upper epidermis and root endosphere, respectively (Fig. 5c). UDP-glucuronosyltransferases is a family of detoxifying enzymes [95-97], which may help plant to detoxify toxic metabolites like fusaric acid, trichothecenes, fumonisins and enniatins produced by *Fusarium* spp. [40, 98] or other co-infected pathogenic fungi. *CsgD*, the master regulator of *Escherichia coli* biofilm formation pathway, can protect microbes from adverse environmental conditions, thereby enhancing microbial survival [99-101]. Although the taxonomic and functional data between healthy and diseased pepper microbiomes provided evidence for plants “cry for help” strategy, culture-based experiments would be required to verify such hypothesis; the enriched “beneficial bacterial strains” should be isolated and tested for their disease suppression effects *in vivo*.

Metagenomic data showed that FWD significantly decreases the functional diversity of KO, COG, and Resfam profiles of microbiome in stem upper epidermis, but not in root endosphere. The reduction in functional diversity could be largely due to the drop in microbial diversity. A number of experimental studies have demonstrated the importance of biodiversity for ecosystem functioning [102-105]. Similarly, our results showed that greater microbiome diversity in healthy plants could ensure the better performance in multiple ecosystem functions. Microbiome communities of greater diversity tend to be more complex, and possess greater functional redundancy and interkingdom associations [48]. In contrast, pathogen invasion could lead to lower microbiome diversity and functionality as a result of disease-induced inhibition of plant photosynthesis [106] and water physiological characteristics [87]. In our study, the relative abundance of alkaline phosphatase gene *phoD*, which is responsible for the recycling of organic phosphorus, was dropped in the diseased plant root endosphere, suggesting that the disease effects plant phosphorus absorption [107]. Greater functional variance in stem upper epidermis than in root endosphere may also reflect the density of microbes surrounding each plant organ, which can be vastly greater for roots [35].

Conclusion

Our investigations improve the understanding of the pepper microbiome assembly and functions under FWD using both below and aboveground parts of the host. Our results demonstrate that host compartment had the strongest effects on the bacterial and fungal microbiome assembly, followed by FWD and sampling site. Fungal communities are more sensitive to FWD than the bacterial communities, and the fungal taxa play a more important role in the diseased co-occurrence interkingdom network. However, fungal communities are less affected by FWD in reproductive than vegetative compartments.

The compartments of diseased pepper plant may recruit beneficial bacteria, which could provide protective functions to host plants. The findings have advanced our understanding on plant-microbe interactions and species coexistence mechanisms. By gaining a better understanding of microbiome

assembly and functions, it would be possible to manipulate microbiomes to improve the resistance of plant to pathogen invasion.

Declarations

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Authors' contributions

LC and MG designed the study. MG and AMZ collected samples. MG and CX conducted the laboratory and data processes. MG and MMW involved in the pathogen strains isolation. MG, LC, CX, CG, CKT, and XZ wrote the manuscript.

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Availability of data and materials

The raw sequencing data have been submitted to the NCBI Sequence Read Archive (SRA) database under the accession number PRJNA667302 (16S), PRJNA667299 (ITS), and PRJNA667562 (metagenomic).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors have no conflicting interests related to this manuscript.

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Figures

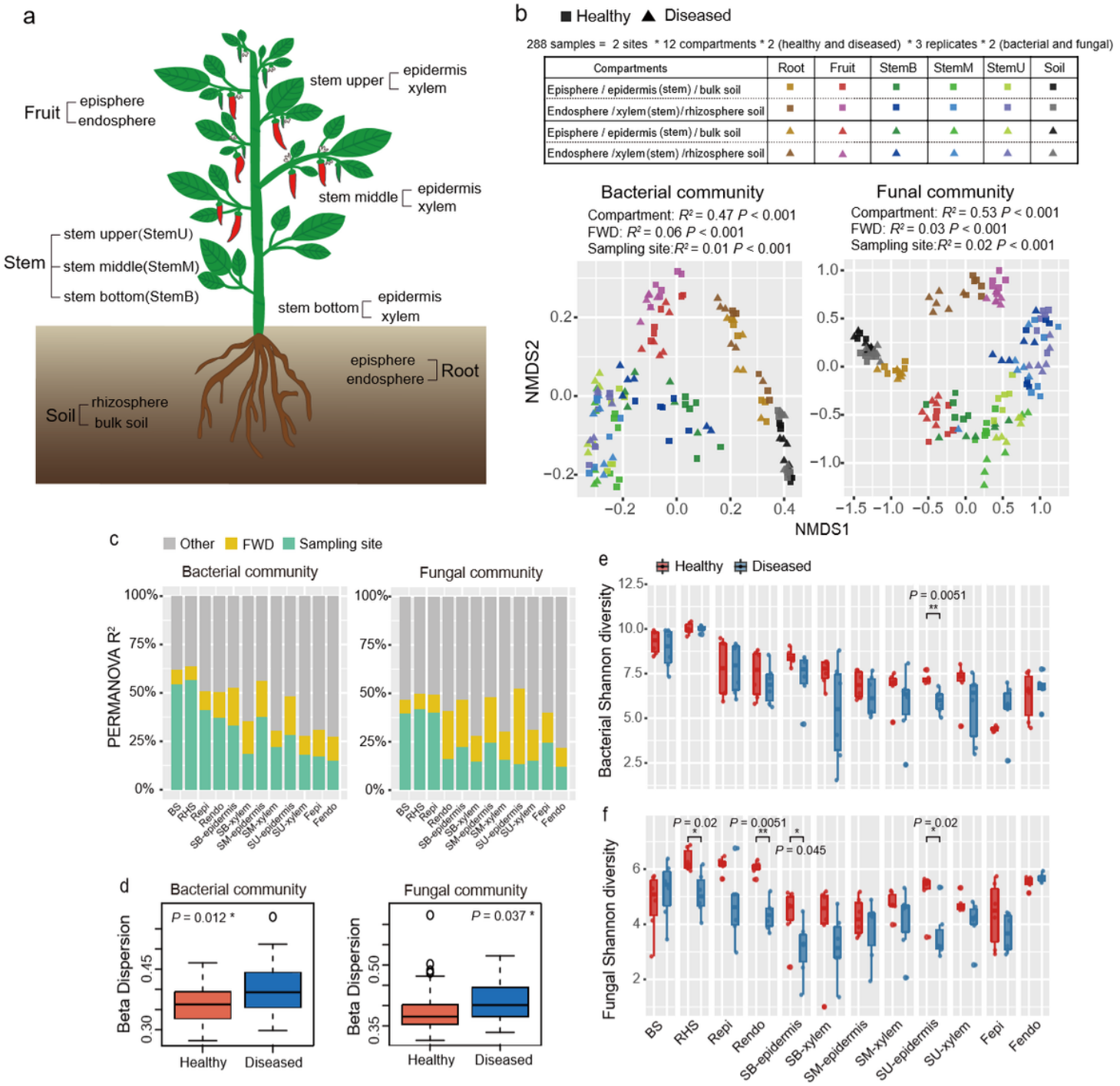
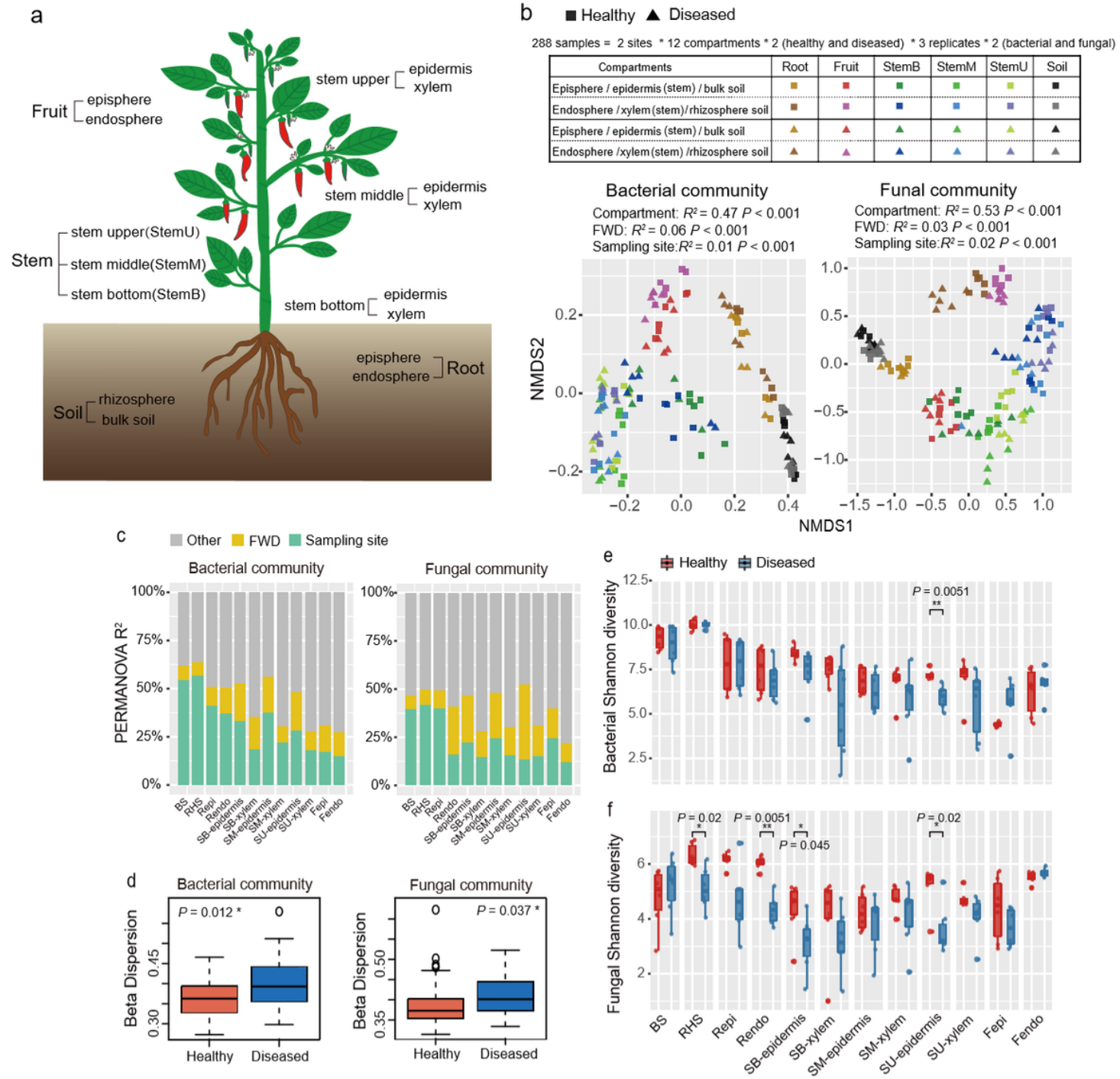


Figure 1

Assembly of pepper bacterial and fungal communities. (a). Diagram of a pepper plant and associated above and belowground compartments, including soil, root, stem, and fruit. (b). Nonmetric multidimensional scaling (NMDS) ordinations of Bray-Cutis dissimilarity matrices with permutational analysis of variance (PERMANOVA) showing significant association of bacterial (left) and fungal (right) community composition with, in order of importance, compartment (R2 = 0.47 for bacteria and R2 = 0.53 for fungi), Fusarium wilt disease (FWD, 0.06 and 0.03) and sampling site (0.01 and 0.02). (c). Contribution of FWD and sampling site on the variation of bacterial (left) and fungal (right) community in

single compartment, based on PERMANOVA, showing that FWD explained higher variation of fungal community than bacterial community in most compartments. (d). Bray-Cutis dissimilarity showing higher dissimilarity in diseased plant than in healthy plant in both bacterial (left) and fungal (right) communities based on betadispersion analysis. (e-f). Shannon diversity of healthy (red color) and diseased (blue color) plant in bacterial and fungal community showing that, FWD decreases Shannon diversity in stem bottom and upper epidermis, root endosphere, and rhizosphere soil fungal community significantly ($P < 0.05$), as well as Shannon diversity in stem upper epidermis bacterial community ($P < 0.05$).



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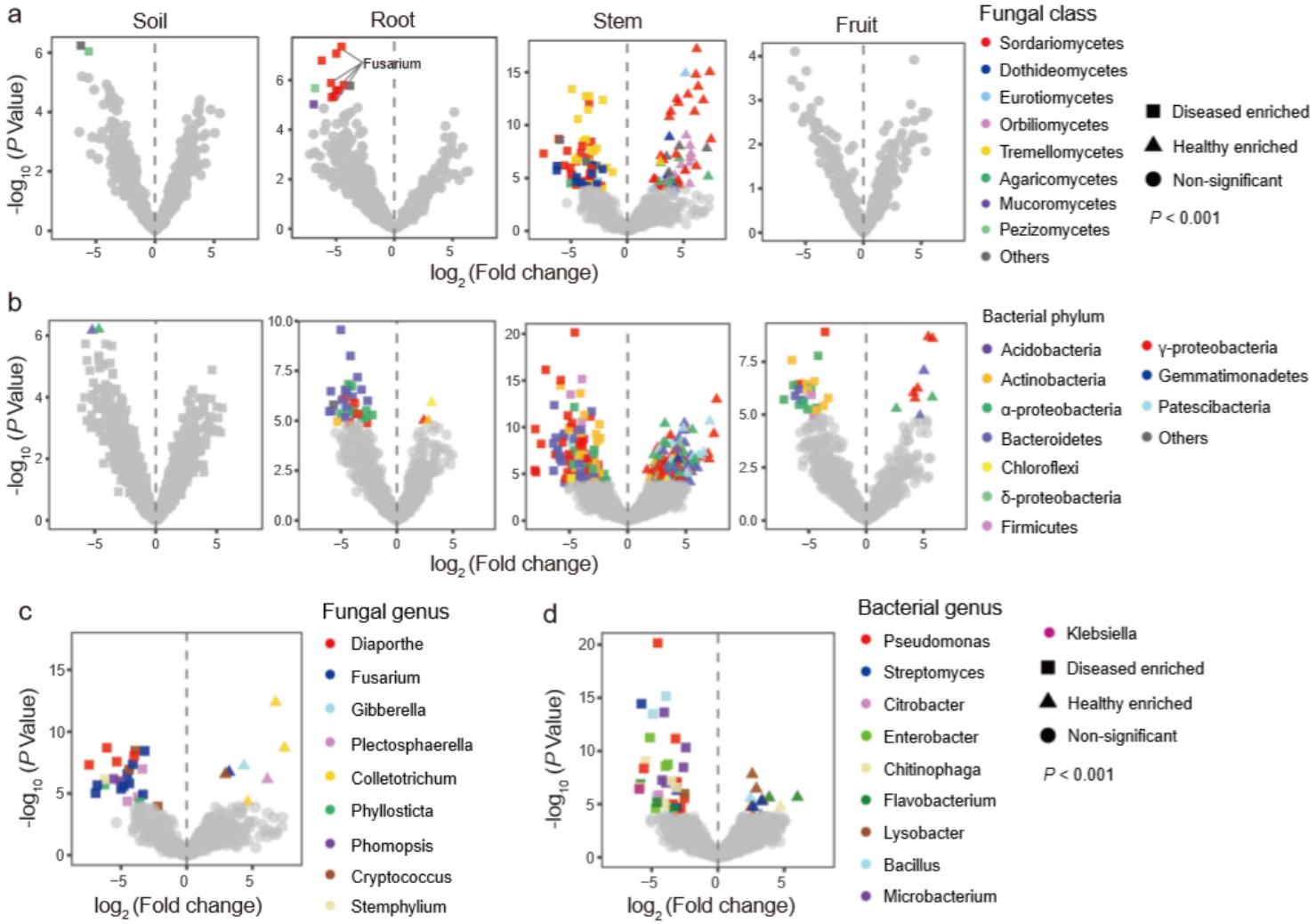
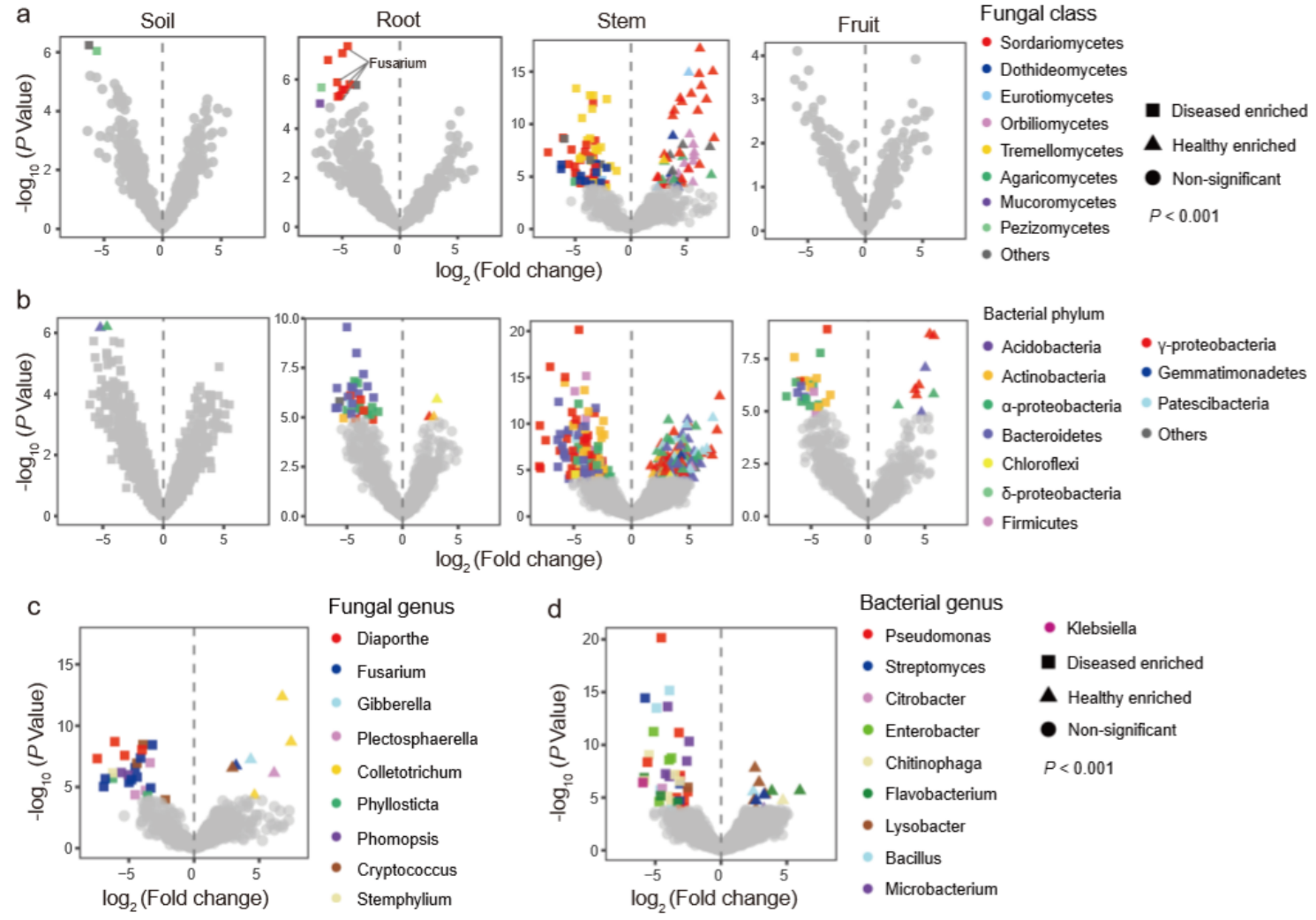


Figure 2

Different organs of healthy and diseased plant were enriched for certain OTUs. (a). FWD effects on fungal OTU abundance. Note the higher abundance of Tremellomycetes in diseased stem, and the functional guild information is presented in Fig S6b. The symbol shape corresponds to healthy (triangle) and diseased (square) enriched OTUs, respectively. (b). FWD effects on bacterial OTU abundance. (c). Abundance of plant pathogenic fungi significantly increased in diseased plant (P < 0.001). (d). Abundance of potential beneficial bacterial taxa significantly increased in diseased plants (P < 0.001). Note that results of these bacterial taxa in root, stem, and fruit are presented in Fig S6c.



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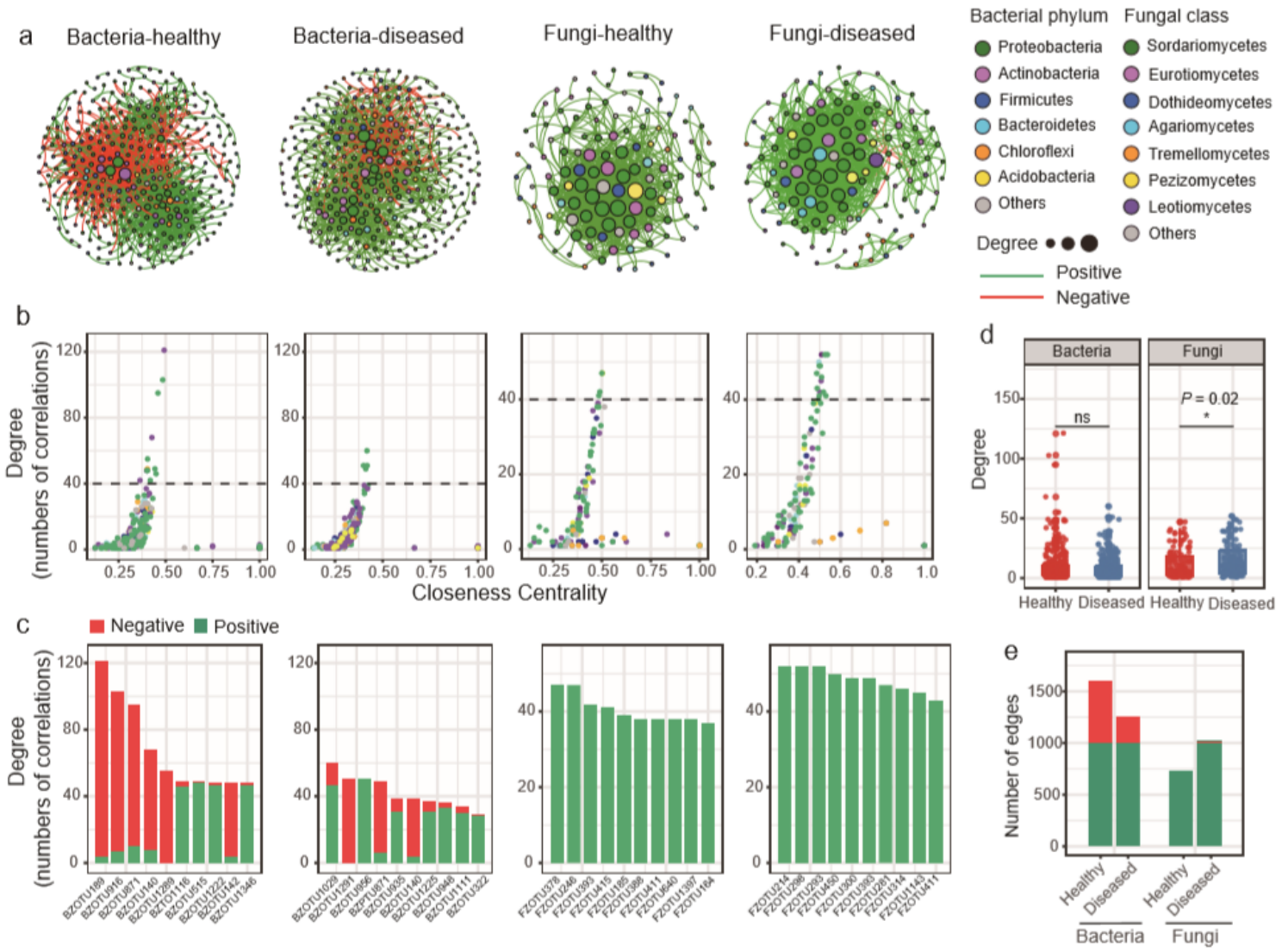


Figure 3

The intra-kingdom co-occurrence networks. (a). Intra-kingdom co-occurrence networks showing a higher number of nodes and edges in bacterial networks than fungal networks. The nodes were colored according to the bacterial phylum and fungal class. The sizes of the nodes are according to the degree of connection. The color of edges represents positive (green) and negative (red) correlations. (b). Comparison of node-level topological features (degree and closeness centrality) showing that the edges of hub nodes had higher degree and centrality values (closeness). The taxonomic information of hub OTUs is presented in Table S10. (c). Degree and interaction type of the top 10 hub nodes in four networks showing higher number of negative correlations in bacterial than fungal networks. The degree (d) and edges (e) of bacterial and fungal taxa in the healthy and diseased networks showing that the healthy bacterial network has higher complexity (based on the degree and edges) than the diseased network, while the opposite pattern in the fungal network. Significance of difference was determined by nonparametric Kruskal Wallis tests.

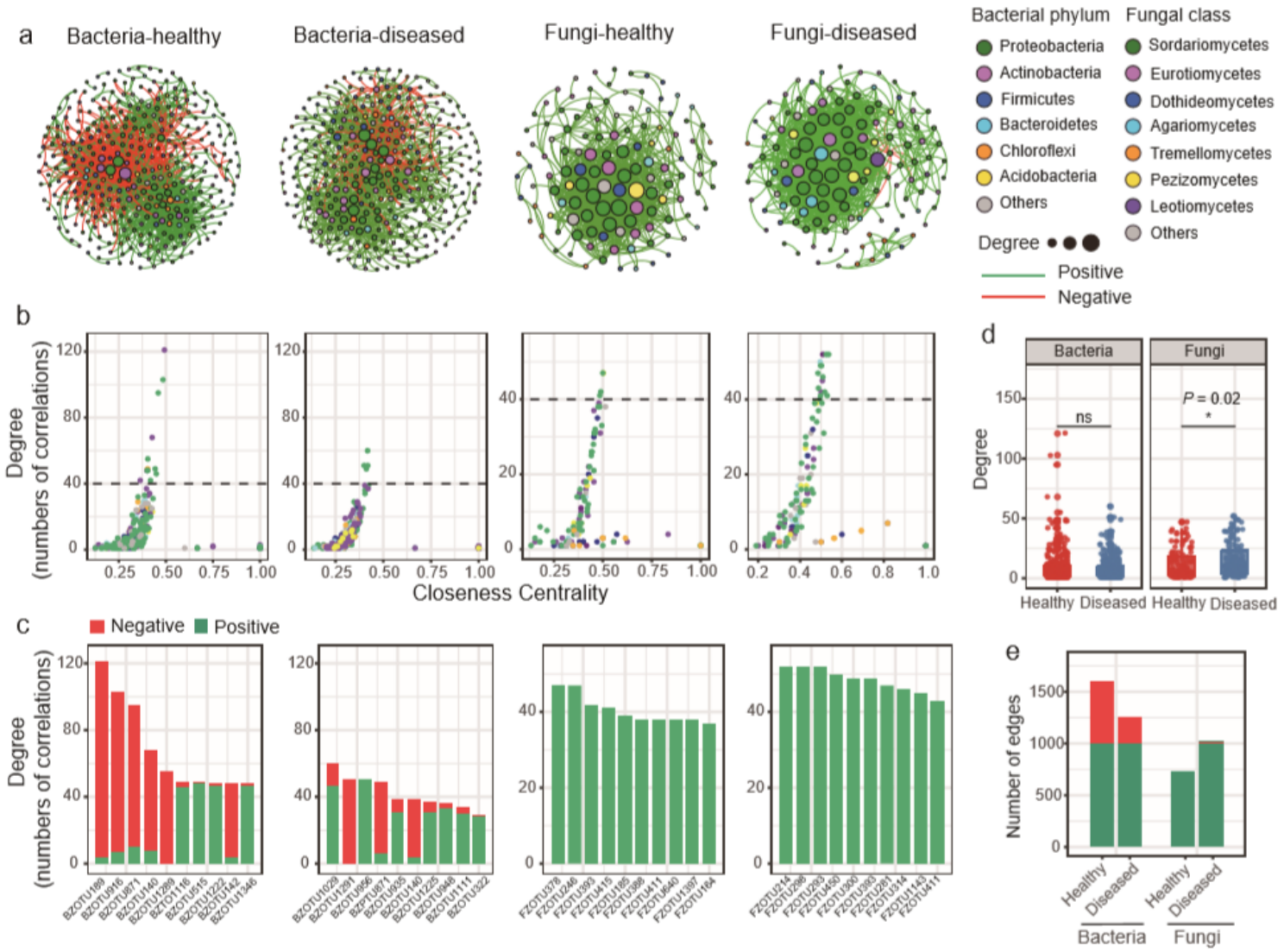


Figure 3

The intra-kingdom co-occurrence networks. (a). Intra-kingdom co-occurrence networks showing a higher number of nodes and edges in bacterial networks than fungal networks. The nodes were colored according to the bacterial phylum and fungal class. The sizes of the nodes are according to the degree of connection. The color of edges represents positive (green) and negative (red) correlations. (b). Comparison of node-level topological features (degree and closeness centrality) showing that the edges of hub nodes had higher degree and centrality values (closeness). The taxonomic information of hub OTUs is presented in Table S10. (c). Degree and interaction type of the top 10 hub nodes in four networks showing higher number of negative correlations in bacterial than fungal networks. The degree (d) and edges (e) of bacterial and fungal taxa in the healthy and diseased networks showing that the healthy bacterial network has higher complexity (based on the degree and edges) than the diseased network, while the opposite pattern in the fungal network. Significance of difference was determined by nonparametric Kruskal Wallis tests.

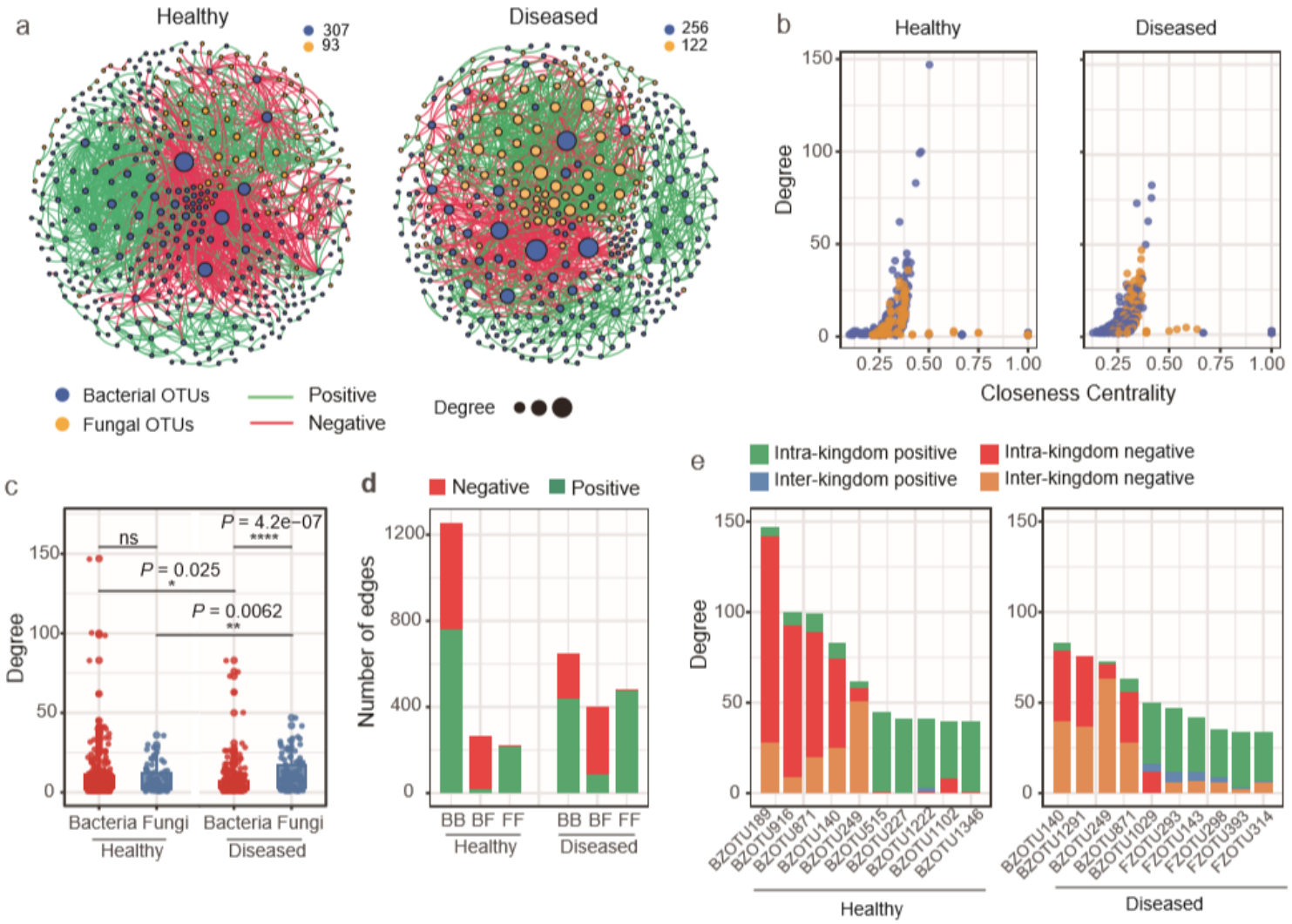
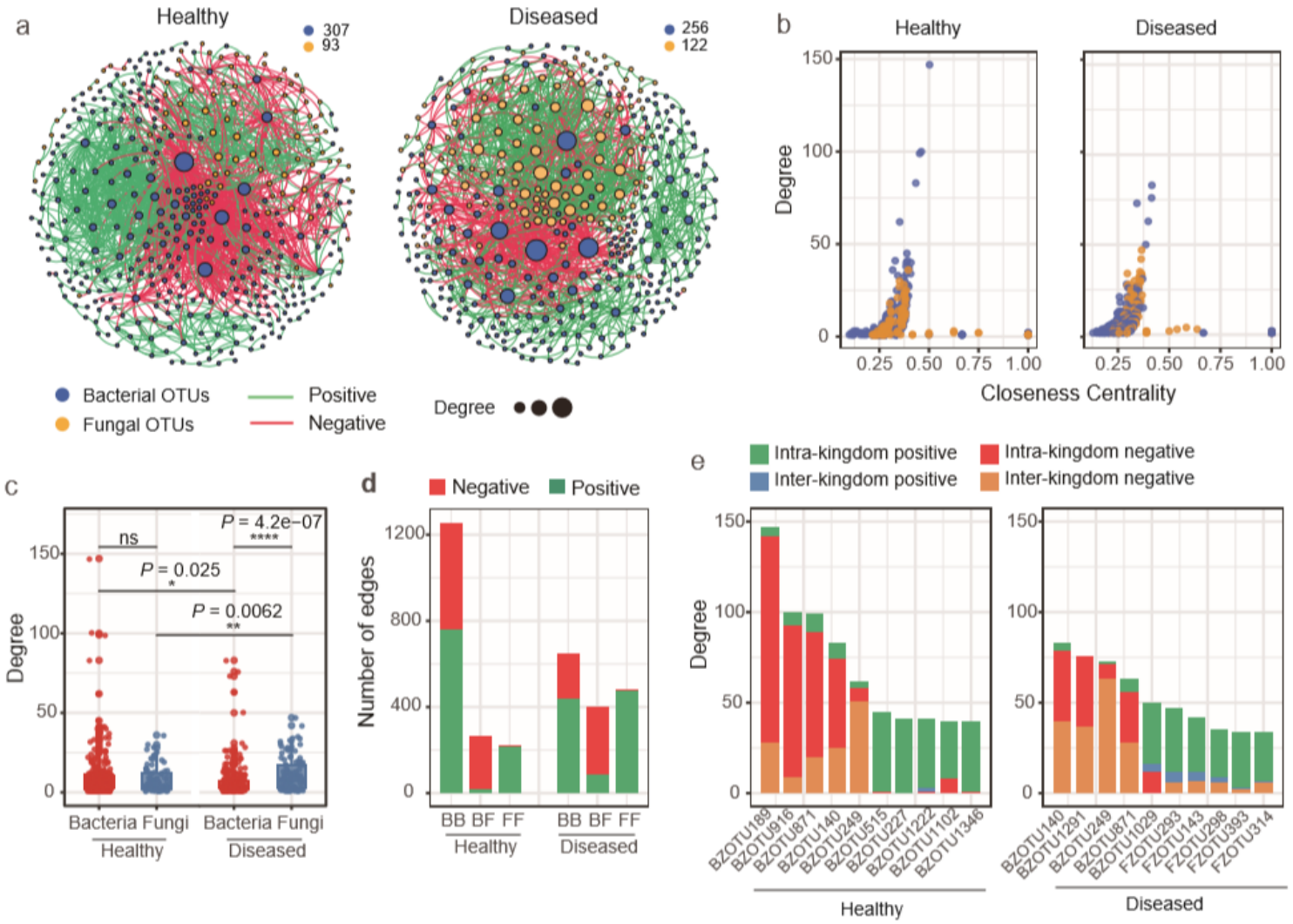


Figure 4

The interkingdom co-occurrence networks. (a). Networks performed with the kingdom of bacteria and fungi together in healthy (left) and diseased (right) plant showing higher number of fungal taxa (orange color) but lower number of bacterial taxa (blue color) in diseased network, when compared with the healthy network. The networks in soil, root, stem (upper, middle, and bottom), and fruit presented in Fig S7. (b). Comparison of node-level topological features (degree and closeness centrality) showing that the edges of hub nodes had higher degree and centrality values (closeness). The top 10 hub species belonged to bacterial taxa in the healthy network, while the fungal taxa account for half number of top 10 hub species in the diseased network. The taxonomic information of hub OTUs is presented in Table S10. (c). Degree of bacterial and fungal taxa in healthy and diseased network. Significance of difference was determined by nonparametric Kruskal Wallis tests. (d). Number of correlations with bacteria-bacteria (BB), bacteria-fungi (BF) and fungi-fungi (FF) in healthy and diseased network. Green and red color of edges and column indicate the positive and negative correlations, respectively. (e). Degree and interaction type of the top 10 hub nodes in healthy (left) and diseased (right) networks. The intra-kingdom correlation means BB or FF, and the inter-kingdom correlation means BF.



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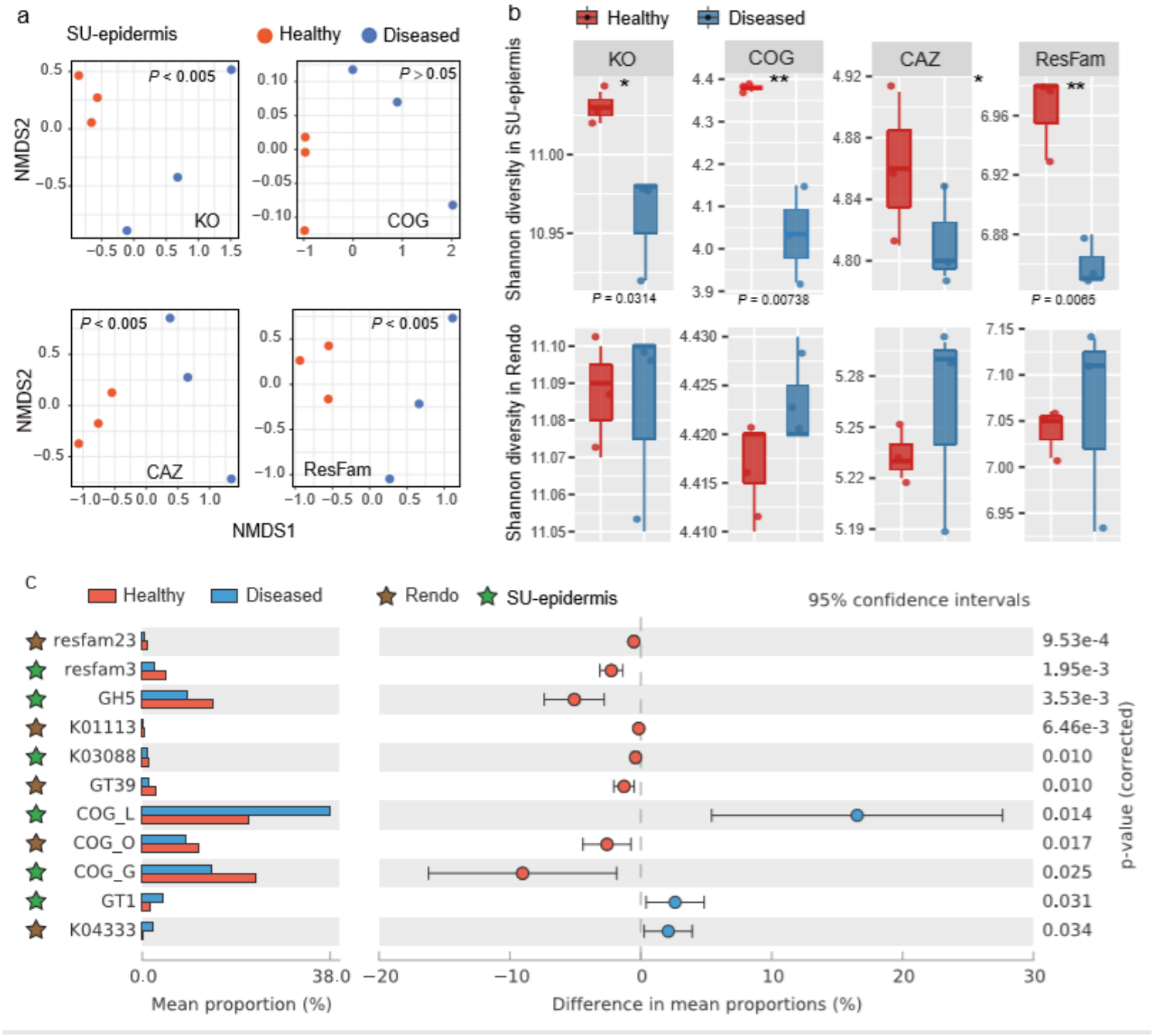
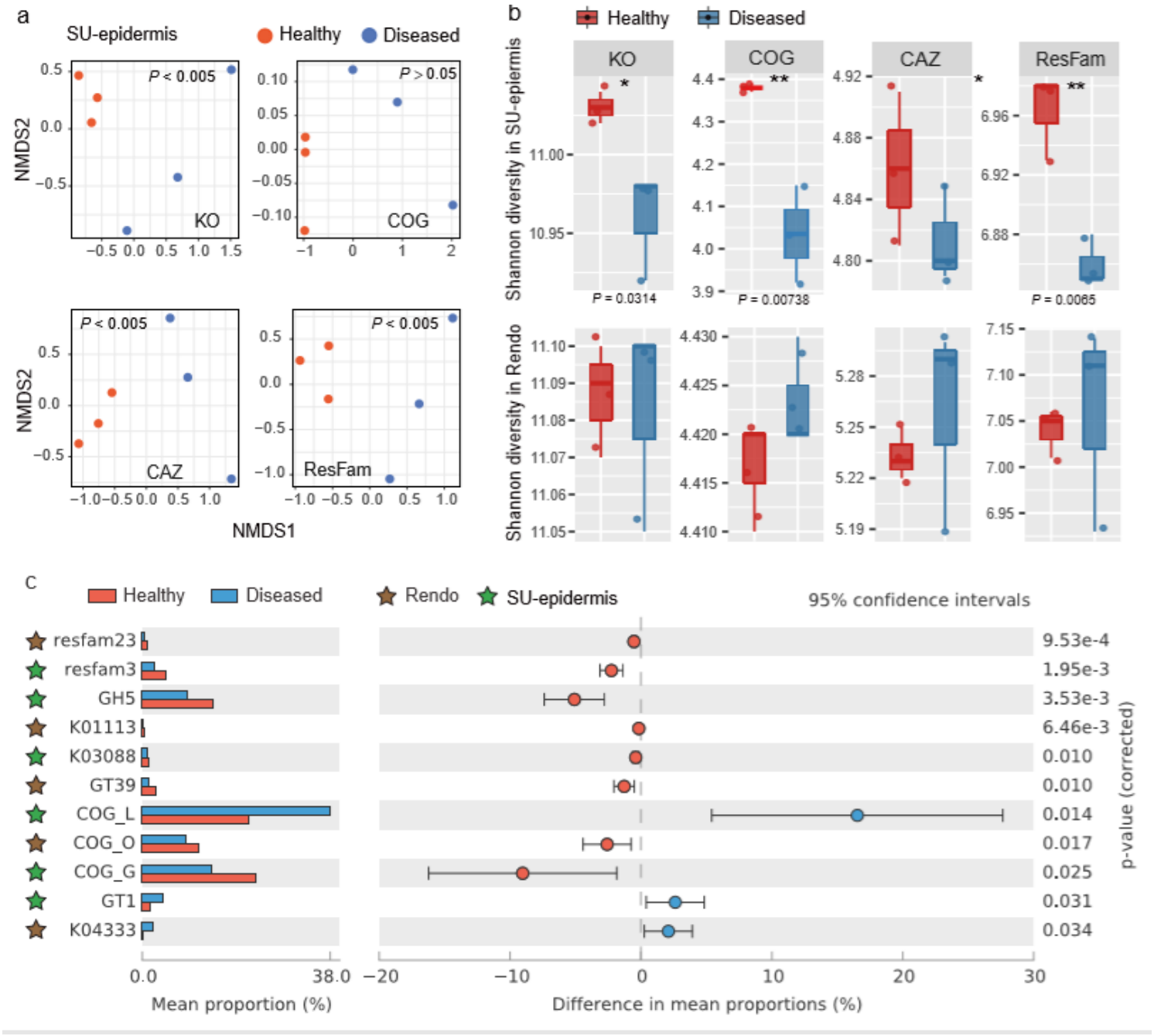


Figure 5

Functional diversity and different functions between healthy and diseased plant based on KO, COG, CAZ, and ResFam functional profiles. (a). NMDS ordinations of functional genes based on bray-cutis dissimilarity with beta dispersion of healthy and diseased stem upper epidermis compartment showing significant association of KO, CAZ, and ResFam, but not in root endosphere (showing in Fig S8a). (b). FWD significantly decreased the functional diversity of KO (P = 0.0314), COG (P = 0.0074), and Resfam (P = 0.0065) profiles in stem upper epidermis microbiome, but showed no significant effects in root endosphere microbiome (P > 0.05). (c). Differential abundance analysis of functions between healthy (red color) and diseased (blue color) plant.



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Supplementary Files

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