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Long-term sod-based rotation promotes beneficial root microbiomes and increases crop productivity

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Abstract

Cotton root microbiomes were investigated in two long-term rotation systems established in 2000, a bahiagrass (*Paspalum notatum* Flugge)-bahiagrass-peanut (*Arachis hypogaea* L.)-cotton (*Gossypium hirsutum* L.) rotation (sod-based rotation, SBR) and a peanut-cotton-cotton rotation (conventional rotation, ConR), from 2017 to 2019. Our results demonstrate that bacterial communities were primarily structured by interannual variability, while fungal alpha and beta diversity were significantly affected by both rotation and interannual variability, with greater fungal diversity and distinct fungal communities in SBR compared to ConR across three sampling years. Cotton roots in SBR also harbored more complex and stable microbial networks. These increased resistance to environmental changes driven by interannual variability, such as temperature and precipitation. Beneficial microbial communities (e.g., Opitutaceae, Pseudonocardiaceae, Rhizobiaceae, Bacillaceae, Comamonadaceae, Serendipitaceae, and Glomeraceae) that may promote plant growth, improve tolerance to abiotic stress, and enhance pathogen defense were associated with cotton roots in SBR, along with fewer pathogenic microbes. These beneficial microbial communities (core microbiomes) together with complex and stable microbial networks were significantly and positively correlated with cotton yield across three sampling years, suggesting that long-term conversion to SBR shaped root microbiomes in a way that increased cotton productivity. This study improves our understanding of the microbial mechanisms that underlie the agronomic and economic benefits observed when integrating perennial grasses to diversify the conventional peanut-cotton rotation.

Keywords Interannual variability · Core microbiomes · Co-occurrence networks · Perennial grass · Sustainable cropping system

Introduction

Cotton (*Gossypium hirsutum* L.) is an important crop at the global scale for fiber used in textile and for cottonseed that is rich in high-quality protein (23%) and oil (21%) (Sunilkumar et al. 2006; Qiao et al. 2017). Cotton is grown in over 80 countries worldwide, where it supports more than 20 million farmers in developing countries (Anthony and Ferroni 2012). The USA is the third-largest cotton producer, especially the Southeast USA that accounts for one-third of total US cotton production (Carlisle et al. 2019). In this region, continuous cotton cropping or rotating cotton with closely related crops reduces cotton productivity and quality due to a high incidence of soil-borne diseases and pests (Katsvairo et al. 2009; Acosta-Martínez and Burow 2010; Huang et al. 2013). Integrating 2 years of bahiagrass (*Paspalum notatum* Flugge) after 1 year each of peanut (*Arachis hypogaea* L.) and cotton, referred to as sod-based rotation (SBR), is an economically viable and more sustainable alternative to the conventional peanut-cotton-cotton rotation (ConR) (Katsvairo et al. 2007a; Schumacher et al. 2020; Zhang et al. 2022). This alternative system decreases pressure from some pathogens and pests and ultimately leads to greater yields (Johnson et al. 1999; Katsvairo et al. 2007b; Tsigbey et al.

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2009), although the underlying biological mechanisms of these benefits are poorly understood.

Plant roots host an overwhelming diversity of microbial communities (i.e., bacteria, fungi, and oomycetes), which can interact with their hosts and impact the host health and fitness (Gaiero et al. 2013; Thiergart et al. 2020). These microbial communities, referred to as root-associated microbiomes, consist of the microbes that live around the roots and are directly affected by the root and root compounds (rhizosphere microbiomes), the microbes that live on the root-soil interface (rhizoplane microbiomes), and the microbes that live in root interior (root endosphere microbiomes) (Lundberg et al. 2012; Edwards et al. 2015; Pascale et al. 2019). Although each area may assemble a distinct microbial community (Xiong et al. 2020), some microbes can live across different compartment niches. For example, hyphae of arbuscular mycorrhizal fungi (AMF) can live in plant tissue and extend their hyphal networks in the rhizosphere (Bonfante and Genre 2010). Some plant growth-promoting bacteria (PGPB), such as *Bacillus* spp., are found in the rhizosphere, rhizoplane, and endosphere of wheat and barley (Xiong et al. 2020). In this study, we defined "root-microbiome" as the microbes that live in the rhizoplane area or/and endosphere.

Plants actively recruit and selectively promote beneficial microbes, e.g., PGPB, from surrounding soil by releasing specific compounds in the rhizosphere (Bulgarelli et al. 2012; Lundberg et al. 2012; Peiffer et al. 2013). Accordingly, harnessing beneficial plant-associated microbes is considered to be one of the most promising approaches in sustainable agriculture (Busby et al. 2017; de-Bashan et al. 2020; Singh et al. 2020), and research focusing on the interactions between microbiota and their host (e.g., cotton) is receiving increasing attention (Qiao et al. 2017; Ullah et al. 2019; Wei et al. 2019; Cassán et al. 2020). Simultaneously, microbial inoculants are becoming research hotpots with the concomitant advances of multi-omics and success in the manipulation of host-associated microbiomes, but their efficacy in field conditions is still unpredictable and unreliable due to the resistance of host-associated microbiomes to manipulation (Kaminsky et al. 2019; de-Bashan et al. 2020). Alternatively, assembling beneficial microbes by managing agricultural practices is widely applied in current sustainable agriculture. For example, wilt-resistant cotton cultivars harbor higher relative abundances of beneficial rhizosphere microbes that improve wilt tolerance compared to susceptible cultivars, including Bacillales, Pseudomonadales, Rhizobiales, and *Trichoderma* (Wei et al. 2019). Li et al. (2015) found that healthy cotton plants may assemble more plant-beneficial and disease-suppressive bacterial taxa in the rhizosphere, e.g., Xanthomonadaceae, Comamonadaceae, Oxalobacteraceae, and Opitutaceae, while cotton plants grown in mono-cropped soils were more associated with cotton pathogens (*Fusarium oxysporum* and *Verticillium dahliae*). Integrating cover crops (e.g., barley and switchgrass) into crop rotations can promote the population and diversity of AMF (Jesus et al. 2016; Hontoria et al. 2019). Overall, crop root-associated microbiomes have the potential to mediate host nutrition, development, and immunity as well as allowing rapid responses to environmental changes. Thus, variations in root-associated microbial communities may be an effective indicator of crop health and growth (Fitzpatrick et al. 2018; Singh et al. 2020; Trivedi et al. 2020). However, the long-term effects of different crop rotation systems on the assembly of root microbiomes and their contribution to cotton performance and yield remain poorly characterized.

Recent studies have shown that microbiomes of plant root and rhizosphere in agricultural ecosystems are modulated by multiple factors (Barnes et al. 2016; Yu et al. 2018; Emmett et al. 2020; Xiong et al. 2020). For example, the genotypes and developmental stage of cotton plants along with soil type notably affect the cotton root microbiome (Kumar et al. 2007; Qiao et al. 2017; Wei et al. 2019). The temporal diversity of plants in a given system and changes in environmental variables are also critical drivers in shaping the composition and diversity of the root microbial community (Berg and Smalla 2009; Naylor and Coleman-Derr 2017; Mavrodi et al. 2018). Hartman et al. (2018) reported that the variation in root bacterial communities was associated with cropping management as well. While the effects of agricultural management or/and environmental factors on belowground communities have been investigated, most prior studies only evaluated a single factor at a time, such as cropping systems (D'Acunto et al. 2018), temporal changes (Wu et al. 2016), or irrigation (Mavrodi et al. 2018). Other studies focused on soil microbial communities only, with no relationship to crop productivity (Liu et al. 2017; D'Acunto et al. 2018), or used short-term field trials (Zhou et al. 2017; Hartman et al. 2018). Thus, field studies exploring the long-term effects of diversified crop rotation systems and irrigation on root microbial communities and adaptive differentiation over several years are lacking.

We collected cotton root samples midway through the growing season from SBR and ConR systems across 3 years (2017–2019) and analyzed their root microbiomes using the DNA amplicon sequencing approach given that bacterial community composition at flowering stage is a predictor of crop yield ($R^2 = 12.3\%$; $P < 0.001$; Xiong et al. 2021). Our objectives were to (1) determine the interactive effects of rotation, irrigation, and interannual variability (the difference in sampling year) on the variations in diversity and structure of microbial communities residing in cotton roots; (2) quantify long-term effects of conversion to SBR on the composition and co-occurrence of root microbiomes; and (3) measure the contribution of root microbiota to their host

performance and yield under different rotation systems. We hypothesized that (1) root microbial communities would be more sensitive to irrigation and interannual variability in ConR as compared to SBR; (2) SBR would enhance the complexity of root microbiomes; (3) SBR would assemble more beneficial microbial communities and increase cotton productivity.

Materials and methods

Experimental site and design

The SBR field trial was established in 2000 at the North Florida Research and Education Center, Quincy, Florida (30°32.79′N, 84°35.50′W) (Dourte et al. 2016). The soil is mapped as a Dothan sandy loam (fine-loamy, kaolinitic, thermic Plinthic Kandiudult), including 85% sand, 5% silt, and 10% clay (Schumacher et al. 2020). Two rotation systems were established in 2000 and grown since then: (1) a 4-year bahiagrass-bahiagrass-peanut-cotton rotation, known as a sod-based rotation (SBR) and (2) a 3-year peanut-cotton-cotton conventional rotation (ConR). Each crop phase is present in every year and considered as a treatment. All treatments are arranged in a strip-plot experimental design in each of three blocks, with 128 × 45.7 m^2 plots, and each block has irrigated (irrigation and precipitation) and non-irrigated (precipitation only) treatments. Details of fertilization and trial maintenance can be found in Schumacher et al. (2020). Basic soil chemical properties in each rotation system are shown in Table S1.

Cotton was planted using a two-row Monosem planter at the rate of 13 seeds/m of row between early April and mid-May every year since 2000. For the years relevant to this study (2017, 2018 and 2019), the cotton variety planted was Deltapine® 1646B2XF. Cotton was harvested in October using a two-row Case IH (CNH Industrial America, LLC, Racine, WI, USA) cotton picker from the 3rd, 4th, 7th, and 8th row of each plot (Schumacher et al. 2020). Cotton lint and seed were weighed (two weights per plot) and a 0.9 kg subsample was ginned from each plot to determine percentage of lint content and seed yield.

Root sampling for DNA amplicon analysis

Three cotton phases (two from ConR and one from SBR) were chosen for this study: first-year cotton in the ConR system (C1), second-year cotton in the ConR system (C2), and the single cotton year in the SBR system (CS). All crop phases for both crop rotations in this study are shown in Table S2. Three individual cotton plants were randomly sampled from the subplots of three cotton phases at the flowering stage (June 2017, 2018, and 2019) using a method modified from Bulgarelli et al. (2012). Roots and shoots were cut with sterilized scissors, adhering soil was removed by shaking the roots vigorously and washing them using distilled deionized (DDI) water twice, and lateral roots of every plant were cut into small fragments (2–3 cm in length). The root fragments of three cotton plants that were collected from the same subplot were mixed thoroughly to form a composite sample. In total, there were 54 composite cotton samples: 3 cotton phases (CS, C1, and C2) × 2 irrigation treatments (irrigation vs. rainfed) × 3 sampling years × 3 blocks. From each composite sample, 0.3 g of fragments was transferred to a 2-ml microtube, and the microtubes were shaken twice with 1.5-ml phosphate-buffered saline (PBS) solution for 20 min at 25 r/s before sonication at 400 Hz for 10 min to remove remaining rhizosphere microbes surrounding roots. Each entire sonicated root sample was then transferred to a new 2-ml microtube containing 10 zirconia beads (2.0 mm dia, BioSpec) and two 2.8-mm stainless steel balls, flash-frozen in liquid nitrogen, and stored at − 80 °C prior to root DNA extraction.

Root DNA extraction, amplicon sequencing, and sequence processing

Root tissues were homologized three times after being flash-frozen in liquid nitrogen using a 1600 Mini-G Tissue Homogenizer (SPEX SamplePrep, Metuchen, NJ, USA) at 1400 r/s for 30 s. Root (including endosphere and rhizoplane) DNA was extracted using the CTAB-DNA extraction method, as described in Liao et al. (2014). The two-step PCR approach was used to construct the DNA library targeting fungal ITS and bacterial 16S rRNA for DNA amplicon sequencing (Chen et al. 2021). The target bacterial 16S V3-V4 and fungal ITS1-ITS2 regions were amplified using the primer set of 341F/806R and ITS1F/ITS4, respectively (Brabcová et al. 2016; Trivedi et al. 2016). Sample-specific 10-bp barcode sequences were linked to the 3′end of reverse primers for the second step PCR. The products from each PCR step were purified using the bead-cleanup approach (AMPure-XP, Beckman Instruments, Brea, CA, USA). The quantity of PCR products was measured using a NanoDrop spectrophotometer (NanoDrop™, Thermo Fisher Scientific, Thermo Scientific™, Wilmington, DE, USA). The size and quality of PCR products were verified by screening using 1% (w/v) agarose gels. All amplicons were pooled at equimolar concentrations (10 ng μl^{-1}), and the index sequencing of paired-end 300 bp was performed on an Illumina (Illumina Inc., San Diego, CA, USA) Miseq (v3 300 bp, 13 Gb sequencing capacity) at the Duke Center for Genomic and Computational Biology. The raw sequence data were deposited in the NCBI Sequence Read Archive (http://trace.ncbi.nlm.nih.gov/Traces/sra/) under Study PRJNA600872.

Bacterial 16 s rRNA and fungal ITS data were processed by the QIIME 2 pipeline (Bolyen et al. 2019). After low-quality sequences (Phred quality score $Q < 20$ or a length shorter than 200 bp) were discarded, a total of 3,625,718 bacterial reads (67,143 reads on average) and 2,412,188 fungal reads (44,670 reads on average) with all samples combined were obtained. High-quality data were mapped to operational taxonomic units (OTUs) with 99% identity cutoff values using the "*vsearch*" function (Frøslev et al. 2017). Bacterial and fungal taxonomy at the species level was assigned to OTUs at a 99% identity threshold using the RDP classifier with the Greengenes (Version 2018) and the UNITE databases (Version 8), respectively (McDonald et al. 2012; Větrovský et al. 2020).

Network analysis

Samples in different cotton phases (CS, C1, and C2) were examined separately for rotation effects on root bacterial and fungal networks, after pooling irrigated treatments across three sampling years, with 18 samples in total for each cotton phase. The OTUs found in the samples of each cotton phase were used to construct co-occurrence patterns in bacterial and fungal communities using the Co-occurrence Network (CoNet) inference in Cytoscape (Shannon et al. 2003). To detect all pairwise associations, an ensemble approach including four methods (Spearman correlation, Pearson correlation, Kullback–Leibler dissimilarity, and Bray–Curtis dissimilarity) was applied (Faust et al. 2012; Faust and Raes 2012). Simultaneously, we performed 1000 renormalized permutations and bootstraps to avoid potential false-positive correlations and compositionality biases. P values ($P < 0.05$) obtained from the above four methods were merged using the Brown method, and then adjusted using Benjamini–Hochberg multiple tests to reduce the occurrence of false-positive results (Zhao et al. 2019). The resulting correlation matrix was imported into the Gephi platform (version 0.9.2) and visualized by the Fruchterman Reingold algorithms (Bastian et al. 2009). The statistics tool in Gephi was used to calculate the topological characteristics of bacterial and fungal networks, including average degree (mean number of edges linked to a node), network diameter (largest length between two nodes within a network), average path length (average length of edges within a network), graph density (closeness of a network), modularity (structure of a network), average clustering coefficient (connectedness among nodes within a network), and percentage of negative correlations (proportion of negative correlations in all correlations of a network).

Statistical analyses

All statistical analyses were performed in R (version 3.5) (Hector 2015; Xia et al. 2018). After chloroplast and mitochondria were excluded from the bacterial OTU table, bacterial and fungal OTU tables were rarefied to 41,200 and 11,100 reads, respectively (Fig. S1). The alpha diversity of bacterial and fungal communities of each sample was measured with the Shannon index and the beta diversity was calculated with a principal coordinate analysis (PCoA) of Bray–Curtis distances (package: *vegan*) (Zhang et al. 2017). Shapiro–Wilk's and Levene's test were used to examine the normality of residuals and homogeneity of variance within the treatments, respectively. For variables where assumptions of normality were violated, the data were either square-root- or log-transformed to achieve normality. Linear mixed models (LMMs) were employed to test the effects of irrigation, interannual variability, and rotation on microbial alpha diversity, microbial taxa, and cotton yield, with the field plot number used as a random effect, using the "lmer" function (package: *lmerTest*). If there were significant interactions (interannual variability by rotation or interannual variability by irrigation) in the LMMs, these interactions were analyzed further. When the interannual variability by rotation interaction was significant, rotation effects for each sampling year and effects of interannual variability for each cotton phase were analyzed separately, after pooling irrigated and rainfed treatments together; significant differences were determined by a one-way ANOVA followed by a Tukey's HSD test for post-hoc comparisons. When the interannual variability by irrigation interaction was significant, the effects of interannual variability were determined for irrigated and rainfed conditions separately using a one-way ANOVA followed by a Tukey HSD post-hoc test, and irrigation effects were determined with a *t*-test comparing irrigated and rainfed conditions for each sampling year individually, after pooling rotation systems. If only the main effect (i.e., interannual variability or rotation) was significant, differences among treatments (i.e., sampling years after pooling rotation and irrigation, and cotton phases after pooling sampling years and irrigation) were determined using a Tukey HSD post-hoc test. Results were considered significant when $P < 0.05$.

A two-way ANOVA was used to compare the effects of irrigation and interannual variability on bacterial and fungal alpha diversity for each cotton phase taken individually. Permutation multivariate analysis of variance (PERMANOVA) was used to test the extent to which variations in factors (irrigation, interannual variability, and rotation) impact root bacterial and fungal communities using the "adonis" function (packages: *vegan*) followed by the corresponding Bray–Curtis distance calculation (Bell et al. 2014). Analyses of similarity (ANOSIM) of the Bray–Curtis distance based on 999 permutations were used to compare the

dissimilarities between sampling years and cotton phases using the "anosim" function (package: *vegan*), based on the main effects of interannual variability and rotation on microbial communities. Random Forest (RF) models were used to detect the fifteen most important microbial taxa (> 0.1% mean relative abundance) across different cotton phases in both rotation systems from 2017 to 2019, using the mean decrease accuracy (MDA) in the "randomForest" function (package: *randomForest*) (Belk et al. 2018). Linear discriminant analysis effect size (LEfSe) was used to identify potential bacterial and fungal bioindicators across whole taxonomic hierarchies from phylum to genus that were significantly enriched in specific sampling year under each cotton phase, based on the threshold of $P < 0.05$ and a linear discriminant analysis (LDA) score > 2.0 (Segata et al. 2011). We defined microbial taxa detected in both RF models and network analysis (nodes) as core microbiomes, given that RF models can accurately identify microbial biomarkers and nodes in microbial networks that may play an ecological role in agroecosystem functions (Tackmann et al. 2018; Banerjee et al. 2019). Pearson correlations between cotton yield and core microbiomes were computed using the "cor" function (package: *corrplot*), and *P* values were adjusted for multiple testing with the Bonferroni-Holm method.

A structural equation model (SEM) was used to evaluate how rotation and irrigation affected the temporal variation of microbial community diversity, core microbiomes, and microbial networks, and how these subsequently affected cotton yield. Rotation variables were built by assigning the value 2 to CS, 1 to C1, and 0 to C2 based on the level of previous crop diversity. Similarly, irrigation variables were created by assigning the value 1 to irrigated and 0 to rainfed treatments. Bacterial and fungal diversity were determined by their corresponding alpha diversity (Shannon index). Core microbiomes were reduced in dimensions by nonmetric multidimensional scaling (NMDS), with the variance of core microbiomes being represented by the first axis of the NMDS (Zhao et al. 2019; Zhang et al. 2020b). The microbial network was represented by the sum of average degree of bacterial and fungal networks. Cotton lint yield was used to represent cotton yield. Variables (rotation, irrigation, average degree, and cotton seed) were standardized by *Z* transformation (mean = 0, standard deviation = 1) using the "scale" function. SEM was fitted by maximum likelihood estimation using the "lavaan" package in R (Rosseel 2012). The SEM fit was determined by a non-significant Chi-square test ($P > 0.05$), the goodness-of-fit-index (GFI > 0.90), and root mean square error of approximation (RMSEA < 0.05) (West et al. 2012).

Results

Cotton yield

There was a significant interannual variability by rotation interaction for cotton seed and lint yield (Table 1). Seed and lint yield were higher in 2018 than in 2017 and 2019 for the cotton phase in the SBR system (CS) and first-year cotton in the ConR system (C1), whereas seed and lint yield was higher in 2018 and 2019 compared to 2017 in second-year cotton in the ConR system (C2) (Fig. 1). Cotton seed and lint yield were greatest in CS and lowest in C2 (2017) or C1 and C2 (2018), with no difference among cotton phases in 2019. Irrigation affected cotton lint yield but not seed yield, with greater cotton lint yield in irrigated plots (1820 ± 36 kg/ha) as compared to rainfed plots (1742 ± 39 kg/ha), regardless of interannual variability or rotation.

Root microbial diversity and community composition

There was a marginally significant ($P = 0.08$) irrigation by interannual variability interaction for bacterial alpha diversity, and no other significant main effects or interactions (Shannon index; Table S3). Under irrigated conditions, bacterial alpha diversity was significantly greater in 2017 compared to 2019 (Fig. 2A), but no difference among years was observed in rainfed conditions. There was no statistical difference in bacterial alpha diversity between irrigated and rainfed plots within an individual sampling year. In contrast, interannual variability and rotation had a significant effect on fungal alpha diversity, with no interaction between them (Table S3). Fungal alpha diversity was greater in 2019 compared to 2017 and 2018, and CS exhibited higher fungal alpha diversity than C1 and C2 (Fig. 2B).

Using PCoA plots of Bray–Curtis distances, PERMANOVA, and ANOSIM analyses to determine the effects

Table 1 Effects of irrigation (I), interannual variability (IV), and rotation (R) on cotton seed and lint yield based on a linear mixed model

		I	IV	R	I×IV	I×R	IV×R	I×IV×R
Seed yield	F	1.19	24.95	11.17	0.44	0.27	5.71	0.47
	P	0.29	**< 0.001*****	**< 0.001*****	0.65	0.79	**< 0.001*****	0.76
Lint yield	F	4.85	49.52	13.54	0.62	0.73	6.05	1.21
	P	**0.03***	**< 0.001*****	**< 0.001*****	0.54	0.49	**< 0.001*****	0.32

*, **, and *** indicate significance at $P < 0.05$, 0.01, and 0.001, respectively

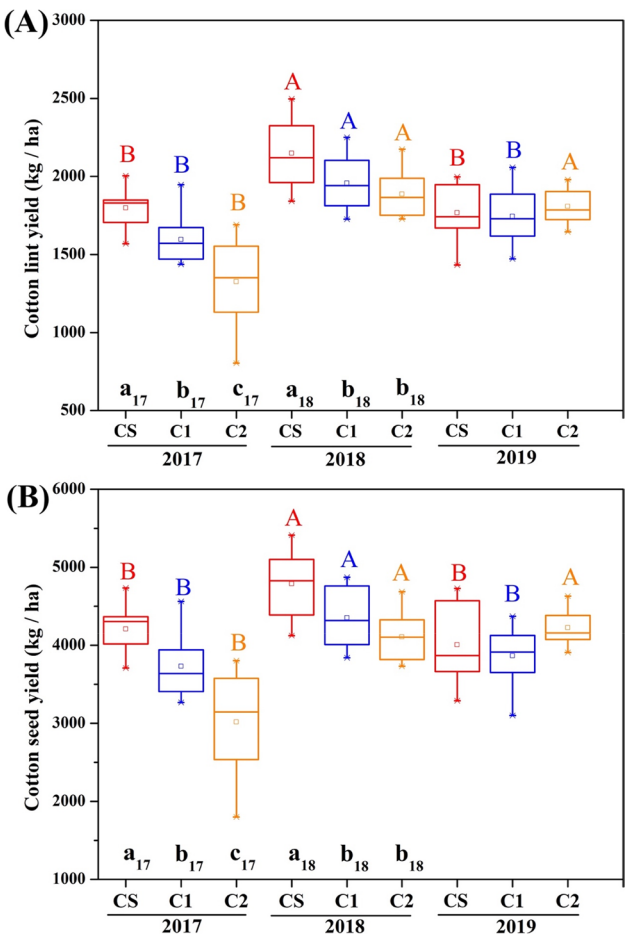

Fig. 1 Cotton lint (**A**) and seed (**B**) yield under different cotton phases in both crop rotation systems from 2017 to 2019. Only significant differences observed in cotton seed and lint yield (Table 1) are labeled with letters. Uppercase letters above boxes indicate significant differences among sampling years within a cotton phase using a one-way ANOVA followed by a Tukey test for post-hoc comparisons ($P < 0.05$), and different colors indicate different cotton phases in both rotation systems (red: CS, blue: C1, and orange: C2). Lowercase letters below boxes indicate significant differences among cotton phases within a sampling year using a one-way ANOVA followed by a Tukey test for post-hoc comparisons ($P < 0.05$), and subscripts indicate different sampling years. CS: cotton phase in sod-based rotation; C1: first year cotton phase in conventional rotation; C2: second year cotton phase in conventional rotation; I: irrigated; R: rainfed

of different factors on beta diversity, we found that bacterial communities were significantly different among sampling years, with no effect of rotation or irrigation (Fig. 2C; Tables 2 and S4). There were significant variations in fungal communities among sampling years and between CS and ConR systems (Fig. 2D), and no effect of irrigation. Interannual variability and rotation ($R^2 = 0.07$) had similar and weak effects on fungal communities, although interannual variability affected bacterial communities more strongly ($R^2 = 0.25$).

Root microbial biomarkers

Using LEfSe analysis to identify bacterial and fungal bioindicators sensitive to sampling year in each cotton phase across whole taxonomic hierarchies from phylum to genus, we found thirty-one bacterial bioindicators affiliated with five phyla (Acidobacteria, Actinobacteria, Proteobacteria, Bacteroidetes, and Verrucomicrobia) that were affected by sampling year across rotation systems (LDA > 2.0; Figs. 3A, B, C and S2). Specifically, seventeen taxa were enriched in 2017, ten taxa had higher relative abundance in 2018, and four taxa increased in 2019. Compared to CS in which five bacterial bioindicators assigned to Proteobacteria and Bacteroidetes were enriched in 2017 (Fig. 3A), seven bioindicators belonging to phyla Actinobacteria and Proteobacteria were significantly higher in 2017 or 2018 in C1 (Fig. 3B), and twenty-three bacterial biomarkers within phyla Actinobacteria, Proteobacteria, and Verrucomicrobia identified in C2 differed significantly across three sampling years (Fig. 3C). Among these bioindicators, taxa within Proteobacteria were predominant across cotton phases, accounting for about 70% of all taxa retrieved, and 55% in C2 (Fig. S3).

The LEfSe analysis identified 25 fungal bioindicators affiliated with phyla Ascomycota (sixteen taxa), Basidiomycota (six taxa), and Glomeromycota (three taxa) that were significantly affected by interannual variability (LDA > 2.0; Fig. 3D–F). Of these bioindicators, nineteen were enriched in 2019, five were significantly increased in 2017, and one had higher relative abundance in 2018. In CS plots, five biomarkers (Clavicipitaceae, *Fusarium*, Ceratobasidiaceae, Tremellales, and Paraglomerales) were sensitive to sampling years. In C1, Chaetothyriales and Auriculariales were enriched in 2019, and Eurotiomycetes had a higher relative abundance in 2017. In contrast, nineteen bioindicators within the phyla Ascomycota, Basidiomycota, and Glomeromycota were identified in C2 plots: Clavicipitaceae, Myrmecridiales, and Acaulospora were significantly higher in 2017, and the others within phyla Ascomycota, Basidiomycota, and Glomeromycota were enriched in 2019 (Fig. 3F).

Random Forest (RF) models were used to evaluate the importance of bacterial and fungal families as potential indicators of rotation systems. Microbial taxa were ranked according to mean decrease accuracy (MDA), and the top 15 were selected as the microbial families that were more sensitive to cotton phases (Fig. 4; Table S5). These bacterial and fungal families, referred to as rotation-specific bacterial and fungal biomarkers, had a total relative abundance ranging from 28 to 37% and 12 to 32% across treatments, respectively (Figs. 4B and D). Among important bacterial biomarkers, nine (Opitutaceae, Blastocatellaceae, Flavobacteriaceae, Polyangiaceae, Pseudonocardiaceae, Rhizobiaceae, Bacillaceae, Alteromonadaceae, and Comamonadaceae) showed higher relative abundance in CS, four (Rhodocyclaceae,

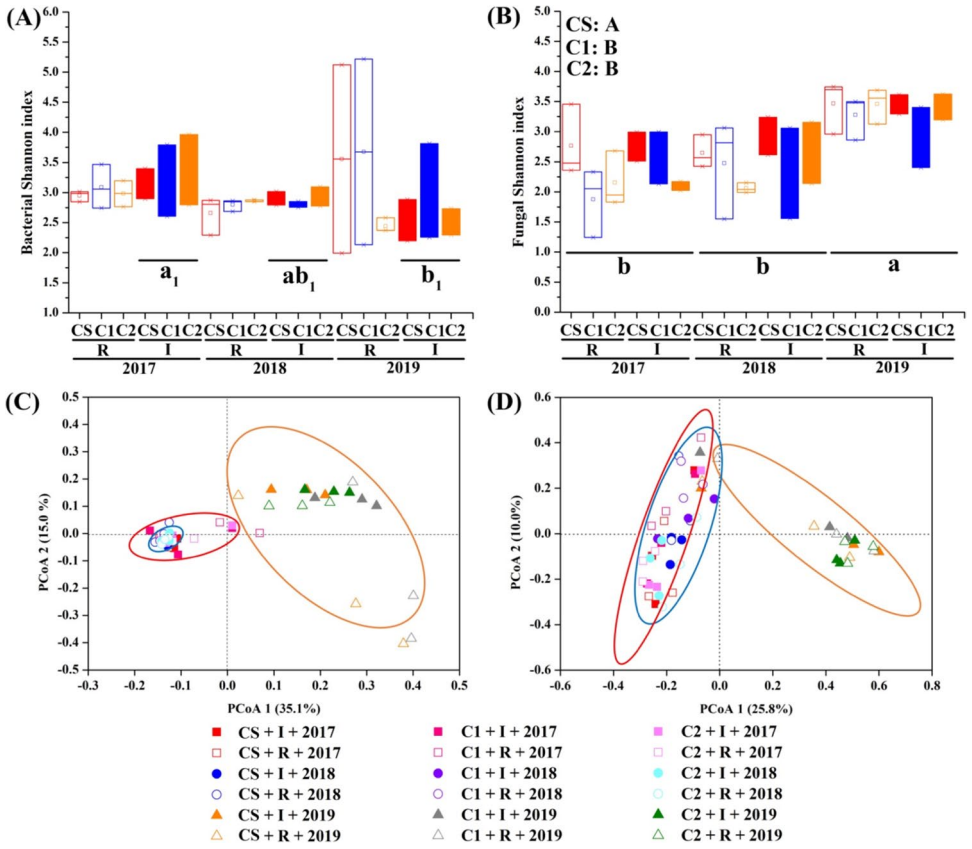

Fig. 2 Alpha and beta diversity of the root bacterial (**A** and **C**) and fungal (**B** and **D**) communities under different crop rotation systems from 2017 to 2019. In A and B, only significant differences observed among groups are labeled with letters and asterisks (see Table S3 for details). Lowercase letters with subscripts indicate significant differences ($P<0.05$) among sampling years using a Tukey test where the interannual variability by irrigation interaction was marginally significant ($P<0.1$). Lowercase letters without subscripts indicate significant differences ($P<0.05$) among sampling years irrespective of irrigation and rotation, using a Tukey test. Uppercase letters indicate significant differences among cotton phases irrespective of interannual variability and irrigation, determined by a one-way ANOVA followed by a Tukey test ($P<0.05$). In C and D, root microbial beta diversity was calculated with a principal coordinate analysis (PCoA) of Bray–Curtis distances. Ellipses include 95% confidence intervals for each sampling year (red = 2017, blue = 2018, and orange = 2019). Comparisons of bacterial and fungal beta diversity among sampling years and cotton phases in rotation systems are shown in Table S4. CS: cotton phase in sod-based rotation; C1: first year cotton phase in conventional rotation; C2: second year cotton phase in conventional rotation. I: irrigated; R: rainfed

Table 2 Effects of irrigation (I), interannual variability (IV), and rotation (R) on the differentiation of bacterial and fungal communities based on PERMANOVA

		I	IV	R	I×IV	I×R	IV×R	I×IV×R
Bacterial community	R^2	0.01	0.25	0.04	0.02	0.02	0.03	0.02
	P	0.49	**0.001*****	0.23	0.27	0.84	0.45	0.65
Fungal community	R^2	0.01	0.07	0.07	0.02	0.01	0.01	0.03
	P	0.85	**0.001*****	**0.001*****	0.45	0.73	0.90	0.47

*, ** and *** indicate significance at $P<0.05$, 0.01, and 0.001, respectively. Details of comparison of microbial community between sampling year are shown in Table S5. I

Acidobacteriaceae, Chthoniobacteraceae, and Burkholderiaceae) had higher relative abundance in C1, and two (Sphingomonadaceae and Parachlamydiaceae) had higher relative abundance in C2 (Figs. 4A and B). For fungal biomarkers, eight biomarkers (Lasiosphaeriaceae, Minutisphaeraceae, Sporocadaceae, Aspergillaceae, Atractosporaceae, Glomeraceae, Stachybotryaceae, and Acaulosporaceae) were enriched in CS, three (Plectosphaerellaceae, Muyocopronaceae, and Gigasporaceae) had higher relative abundance in C1, and four (Serendipitaceae, Magnaporthaceae, Tricholomataceae, and Clavulinaceae) had a higher relative abundance in C2 (Figs. 4C and D).

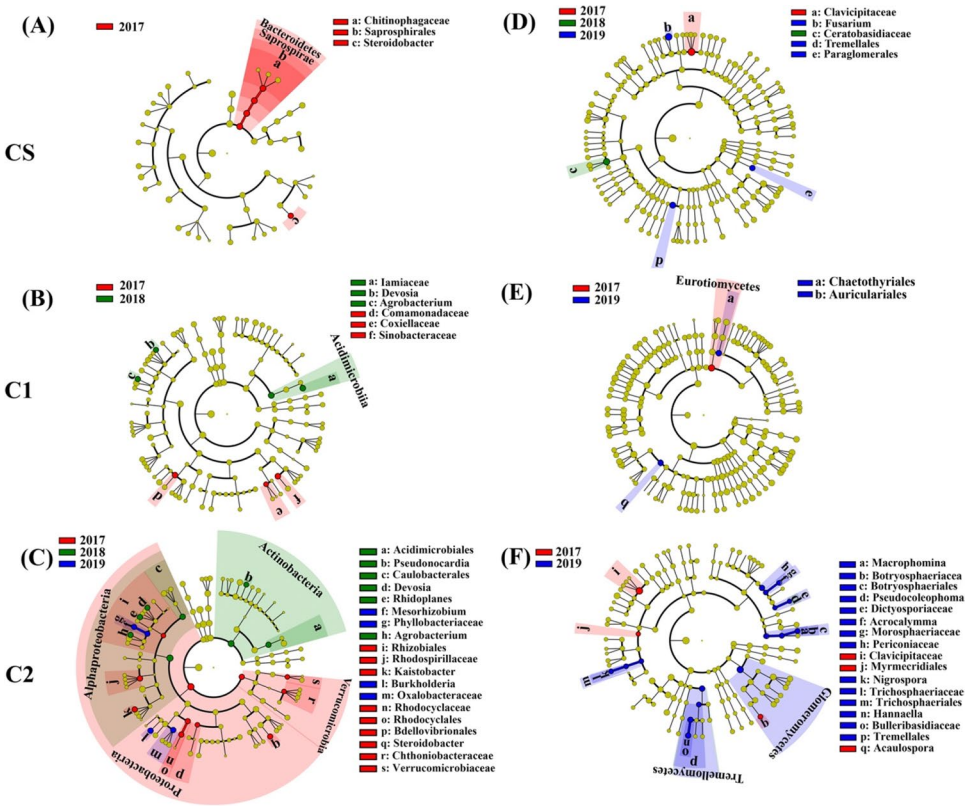

Fig. 3 LEfSe analyses indicating interannual differences of root bacterial (**A**–**C**) and fungal (**D**–**F**) taxa under different cotton phases in rotation systems. The six circular rings in the cladogram represent six taxonomic levels, from inside to outside: supergroup, phylum, class, order, family, and genus, respectively. Nodes on each circular ring represent a taxon. Color nodes (except yellow) indicate taxa with significantly higher relative abundances in a certain sampling year within each cotton phase in both rotation systems (red for 2017, green for 2018, and blue for 2019). The LDA score of each microbial biomarker is shown in Fig. S2. CS: cotton phase in sod-based rotation; C1: first year cotton phase in conventional rotation; C2: second year cotton phase in conventional rotation

Co-occurrence patterns in root bacterial and fungal networks

Co-occurrence networks were constructed to further evaluate root bacterial and fungal networks for cotton phases in SBR and ConR systems (Fig. 5). Topological characteristics of bacterial and fungal networks were computed to determine the co-occurrence patterns in bacterial and fungal communities under different cotton phases. The bacterial network in CS consisted of 38 nodes among taxa at the family level and 195 edges (representing associations among taxa), which were higher than those in C1 (27 nodes and 101 edges) and C2 (30 nodes and 143 edges) (Fig. 5A, C, and E; Table 3). The average degree, modularity, average clustering coefficient, and percentage of negative correlations in CS were considerably higher than those in C1 and C2 (Table 3). However, the taxonomic composition encompassed by the bacterial networks of CS, C1, and C2 was similar (Table S6).

Similar to bacteria, CS had a more complex fungal network compared to C1 and C2, with a higher number of nodes (52) and edges (60) as well as a greater taxonomic composition relative to C1 (9 nodes and 18 edges) and C2 (11 nodes and 10 edges) (Figs. 5B, D, and F; Tables 3 and S6). CS also had higher modularity and average path distance but a lower percentage of negative correlations in the fungal network relative to ConR systems.

Linkages between root microbial diversity, core microbiome, and cotton yield

Pearson correlations between cotton yield and the relative abundance of core microbiomes indicate that cotton seed and lint yield were positively correlated with the relative abundance of bacterial families (Opitutaceae, Pseudonocardiaceae, Rhizobiaceae, Bacillaceae, and Comamonadaceae) and fungal families (Serendipitaceae and Glomeraceae) in CS (Fig. 6). Cotton seed yield had a negative correlation with the relative abundance of Muyocopronaceae in CS. In contrast, there were fewer significant correlations between the relative abundance of core microbiomes and cotton yield in ConR systems: cotton yield was positively correlated to the relative abundance of Rhizobiaceae in C1 and negatively correlated to the relative abundance of Sphingomonadaceae in C2.

The structural equation model constructed to characterize direct and indirect effects of rotation and irrigation on microbial alpha diversity, core microbiomes, microbial network, and cotton yield indicated that rotation (total coefficients = 0.99) had a greater effect on cotton yield than irrigation (total coefficients = 0.08), consistent with the linear mixed model analysis (Fig. 7B; Table 2). Bacterial (total coefficients = − 0.31) and fungal (total coefficients = 0.27) alpha diversity had contrasting impacts on core microbiomes, and only fungal alpha diversity (total

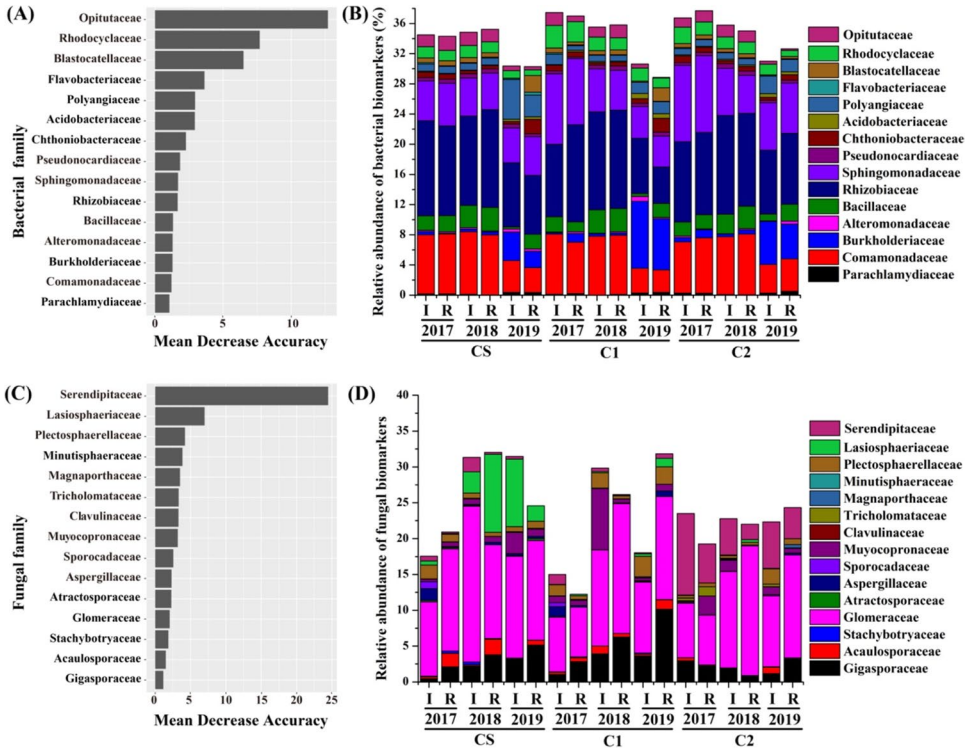

Fig. 4 Random Forest models detecting root bacterial (**A**) and fungal (**C**) biomarkers across crop rotation systems. The top fifteen bacterial and fungal taxa assigned at the family level were determined with the mean decrease accuracy. Stacked barplots show the relative abundance of these top 15 bacterial (**B**) and fungal (**D**) biomarkers under different treatments. CS: cotton phase in sod-based rotation; C1: first year cotton phase in conventional rotation; C2: second year cotton phase in conventional rotation; I: irrigated; R: rainfed

coefficients = 0.27) contributed largely to the microbial network. Fungal alpha diversity (total coefficients = 0.26), core microbiomes (total coefficients = 0.29), and microbial networks (total coefficients = 0.94) were significantly affected by rotation, and they were significant factors in regulating cotton yield ($P < 0.05$; Fig. 7A). In contrast, bacterial alpha diversity had a negative impact on cotton yield (total coefficients = −0.29) and it was unaffected by rotation.

Discussion

The response of root microbiota to different crop rotation systems

In this study, we performed an in-depth comparative analysis of microbial communities residing in cotton roots subject to different agricultural management systems (i.e., long-term crop rotation and irrigation) and interannual variability, observing different responses of bacterial and fungal communities to these factors. While bacterial communities were relatively unaffected by rotation systems, CS exhibited strong effects on fungal diversity and community composition across all sampling years, implying that conversion to the SBR system structured root fungal communities in the long term, probably due to the significant legacy effect generated by the SBR system (Zhang et al. 2021). Bahiagrass, grown in SBR but not ConR, can translocate a greater quantity of fixed C to belowground structures (e.g., root biomass and root mass), and SBR can increase the diversity of C exudates due to crop legacy effects that structure the fungal community of cotton root (Katsvairo et al. 2007c; Dourte et al. 2016; Pausch and Kuzyakov 2018; Zhang et al. 2021), given that fungal groups are the primary consumers of root-derived C (Hugoni et al. 2018; Zhang et al. 2021). Different plant functional groups (i.e., bahiagrass) cause longer lasting changes in the fungal relative to the bacterial community (Hannula et al. 2019, 2020), consistent with the greater relative abundance of fungal biomarkers (e.g., Lasiosphaeriaceae, Minutisphaeraceae, Sporocadaceae, and Atractosporaceae) we found in CS relative to C1 and C2. Some root fungal endophytes can also serve as saprophytes (Põlme et al. 2020; Zhang et al. 2020a) that directly benefit from diverse C sources, and we only detected fungal saprotrophic groups (e.g., Torulaceae, Pyronemataceae, and Ancylistaceae) in CS (Fig. S4). Jach-Smith and Jackson (2018) found that integrating a perennial grass in rotation can also benefit arbuscular mycorrhizal fungal (AMF) communities, consistent with Glomeraceae and Acaulosporaceae enriched in CS. Consequently, it's very likely that the higher plant diversity in the SBR system that combines peanut, cotton, and bahiagrass is what led to higher fungal diversity and a distinct fungal community composition relative to ConR systems that only include peanut and cotton.

Given the higher fungal alpha diversity found in CS, we expect that microbial communities in the SBR system would likely be more resistant to environmental perturbations, such

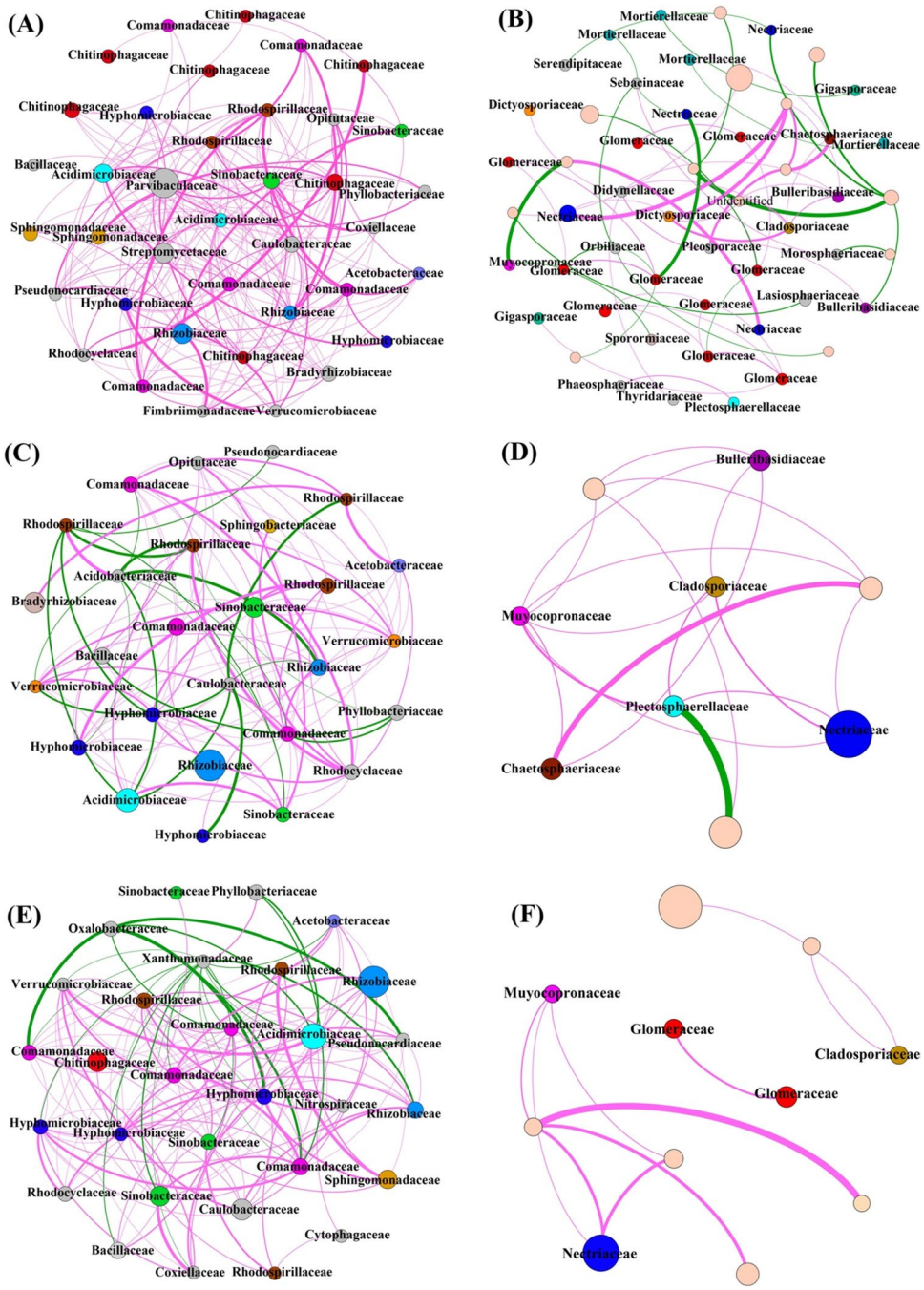

Fig. 5 Bacterial (**A**, **C**, and **E**) and fungal (**B**, **D**, and **F**) co-occurrence networks in cotton roots under CS (**A** and **B**), C1 (**C** and **D**), and C2 (**E** and **F**). Samples for constructing bacterial and fungal networks under each cotton phase have irrigated treatments pooled across three sampling years. Each node represents a taxon at the family level, and node size is proportional to the relative abundance of this taxon. Nodes without taxonomic information in B, D, and F could not be identified. Edge thickness is proportional to the value of each correlation coefficient, and pink and green edges represent positive and negative correlations, respectively. The summary of taxonomic information for each node is shown in Table S6

as the changes in soil moisture, temperature, and precipitation (Isbell et al. 2015). When analyzing the effects of interannual variability and irrigation on alpha diversity of microbial communities in each cotton phase individually (Table S7), interannual variability, but not irrigation, had a significant effect on the alpha diversity of bacterial or/and fungal communities only in ConR systems, especially in the C2 system, and more microbial biomarkers linked to interannual variability were detected in C2 relative to CS. This suggests that root microbial community composition in C2 is more susceptible to changes over years, which could be driven by changes in surrounding environments, e.g., from the fluctuation in temperature and precipitation (Mavrodi et al. 2018). This interpretation is supported by the significant effect of temperature and precipitation on the diversity of microbial communities in ConR systems, despite the small variation in temperature observed among years during the study period (Fig. S6).

As root-associated microbiomes are highly associated with the diversification, development, growth, and fitness of plants (Hardoim et al. 2015; Fitzpatrick et al. 2018; Delaux and Schornack 2021), it is critical to thoroughly understand how microbial associations harbored in crop roots are affected

Table 3 Topological properties of the microbial co-occurrence networks in the different cotton phases in cotton systems

	CS		C1		C2	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
Number of nodes	38	52	27	9	30	11
Number of edges	195	60	101	18	143	10
Average degree	11.842	2.308	8.222	3.625	9.533	1.818
Network diameter	4	8	5	3	4	3
Average path length	1.974	3.090	2.156	1.528	1.869	1.579
Graph Density	0.277	0.045	0.880	0.500	0.329	0.182
Modularity	0.526	0.765	0.394	0.464	0.352	0.338
Average clustering coefficient	0.603	0.305	0.497	0.474	0.537	0.500
Percentage of negative correlations	100%	63.3%	79.2%	94.4%	82.5%	100%

CS, cotton phase in sod-based rotation; *C1*, first year cotton phase in conventional rotation; *C2*, second year cotton phase in conventional rotation

by rotation complexity and how rotation-specific microbiomes affect crop performance. Our results highlight that rotation systems had a significant impact on the network structure of root microbiomes and reveal that CS, found in a more diverse rotation, harbored more complex microbial networks than ConR systems, with a greater number of connected taxa (nodes) and associations among taxa (edges). As previous studies concluded that complex microbial networks were more resistant and resilient to environmental changes than simple networks (Santolini and Barabási 2018; Banerjee et al. 2019), the higher complexity of microbial networks in CS may make the root microbiomes more resilient to environmental changes, e.g., changes in precipitation and temperature (Fig. S6).

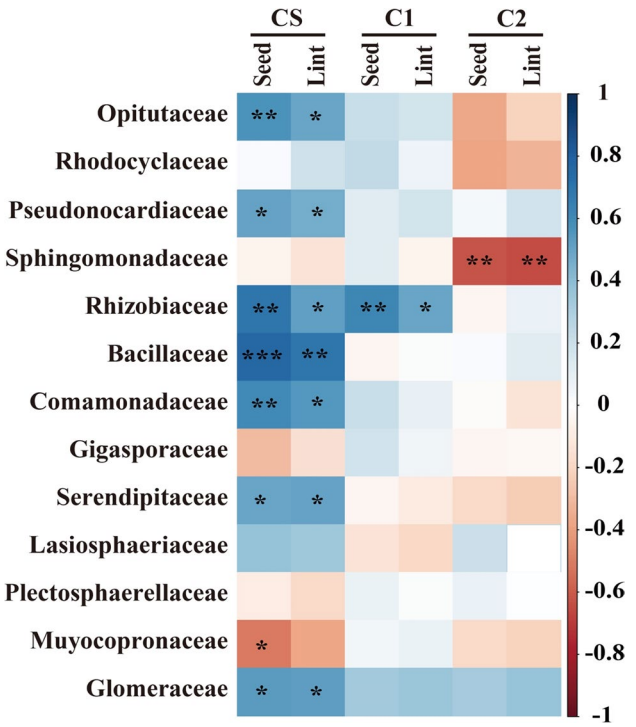

Fig. 6 Heatmap of correlations between cotton yield and core microbiomes in the cotton phases of SBR (CS), and first year (C1) and second year (C2) CR systems across three sampling years. Darker colors (blue is positive, brown is negative) indicate stronger correlations. * and ** indicate significance at $P < 0.05$ and 0.01, respectively, determined by the Pearson correlation coefficient after correction for multiple comparisons using the Bonferroni-Holm method

Rotation-specific root microbial biomarkers/core microbiomes and their roles in crop yield

Most of CS-enriched bacterial biomarkers, e.g., Opitutaceae, Pseudonocardiaceae, Rhizobiaceae, Bacillaceae, and Comamonadaceae, may serve as plant growth-promoting bacteria (PGPB) (Backer et al. 2018; Kumari et al. 2019; Vuko et al. 2020). It has been suggested that taxa belonging to Opitutaceae, Rhizobiaceae, and Comamonadaceae can biologically fix atmospheric N for plant uptake (Glick 2012; Rodrigues and Isanapong 2014; Vuko et al. 2020). Although it is not known if these taxa are able to fix N in cotton roots, they can fix N in non-legume plant roots. For example, OTUs assigned to Opitutaceae sp. (diazotrophic *nifH* communities) were detected in active perennial grass roots during the summer (Gupta et al. 2019). Bahulikar et al. (2020) reported that *Pseudacidovorax intermedius* belonging to Comamonadaceae was one of the dominant *nifH*-expressing taxa in switchgrass roots. In addition, Opitutaceae and Comamonadaceae have the potential to antagonize pathogens at the rhizosphere-cotton root interface (Li et al. 2015). Members of Bacillaceae and Pseudonocardiaceae may colonize and foster interactions with roots of the host (Allard-Massicotte et al. 2016), where they serve as biofertilizers and affect several mechanisms, such as producing growth-stimulating phytohormones (i.e., cytokinins and gibberellins) and siderophore, solubilizing and mobilizing phosphate, inducing plant systemic resistance to pathogens, and lysing fungal mycelia (Platas et al. 1998; Beneduzi et al. 2012; Glick 2012; Strobel et al. 2012). Accordingly, CS may promote the assembly of beneficial bacterial taxa

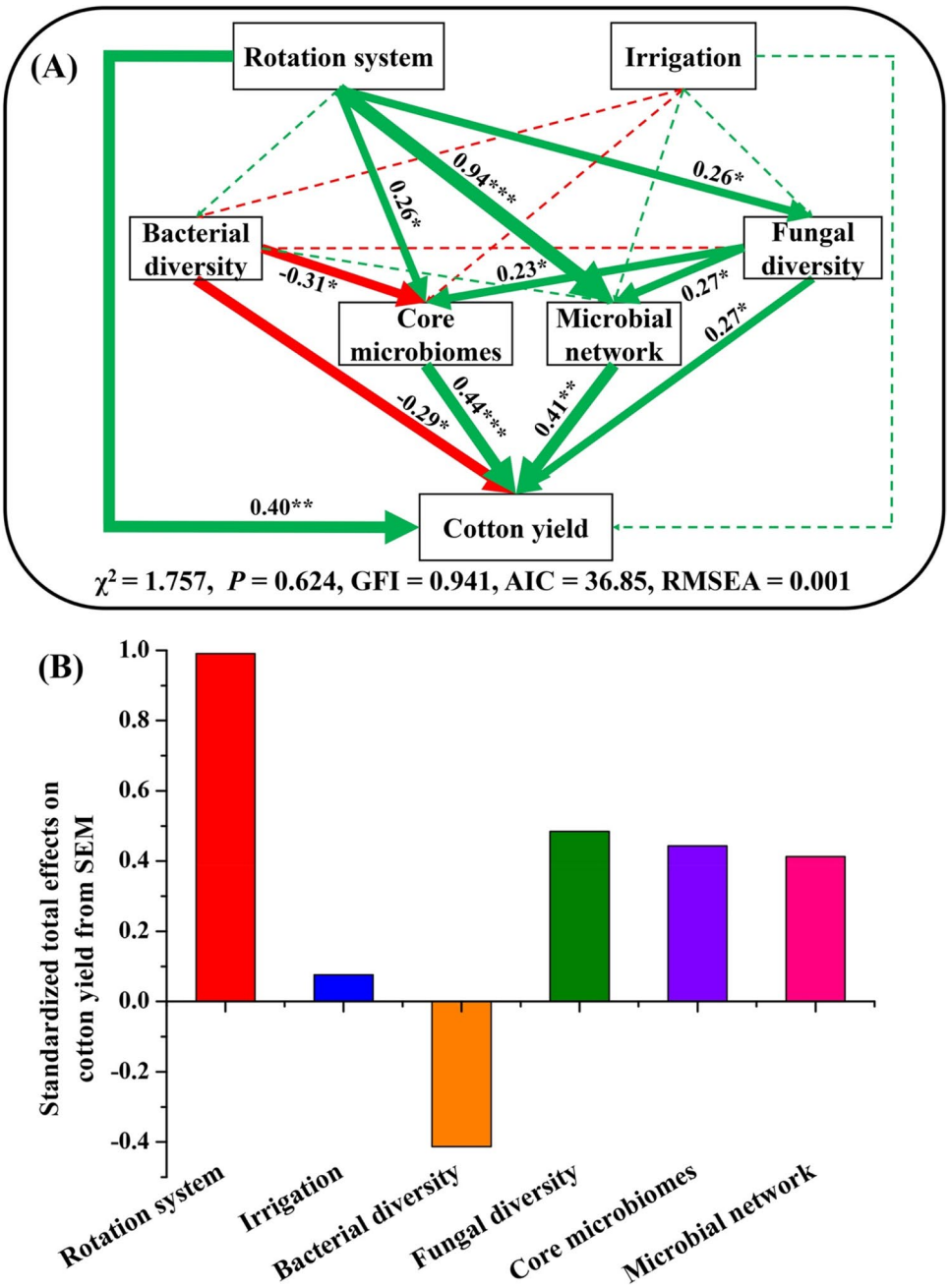

Fig. 7 Structural equation model (SEM) (**A**) illustrating the effects of rotation system and irrigation on interannual variation in cotton root microbiomes and cotton seed yield. Alpha diversity (Shannon index) was used for bacterial and fungal diversity. Rotation variables were built by assigning the value 2 to CS, 1 to C1, and 0 to C2 based on the level of previous crop diversity, and then were standardized by *Z* transformation. Core microbiomes were the taxa detected in Random Forest models and nodes in network analyses, which were reduced in dimensions by NMDS (variance of core microbiomes is represented by the first axis of NMDS). Microbial networks are represented by the sum of average degree of bacterial and fungal networks. Continuous and dashed arrows show significant and nonsignificant relationships between two measured variables, respectively. Green and red arrows indicate positive and negative relationships, respectively. The width of arrows shows the strength of path coefficients. *, **, and *** indicate significance at $P < 0.05$, 0.01, and 0.001, respectively. Standardized total effects (**B**) were calculated as the sum of direct and indirect effects from the SEM

within cotton roots and subsequently support their growth, as microbial adaptation and survival highly depend on physiological conditions of the host once recruited inside plant tissue (Hardoim et al. 2008, 2015). This is consistent with prior studies where disease-resistant and healthy cotton can induce more plant-beneficial (e.g., N-fixing) and disease-suppressive bacterial taxa, including Bacillales, Rhizobiales, Comamonadaceae, and Opitutaceae (Li et al. 2015; Wei et al. 2019). Coincidently, these PGPB taxa were also detected in bacterial networks across SBR and ConR systems and thereby defined as core microbiomes. A greater number of edges associated with these core microbiomes involved in CS-specific bacterial networks, as compared to ConR systems (Table S7), highlight that these core microbiomes may tightly interact with other taxa residing in cotton roots in CS and ultimately play an important ecological role in microbiome functioning (Ma et al. 2016; van der Heijden and Hartmann 2016; Hartman et al. 2018). The recruitment and assembly of these core microbiomes could also promote crop growth by promoting nutrient acquisition, providing bacteria-synthesized compounds to support crop development, and antagonizing the inhibitory effects of various pathogens on crop growth (Fitzpatrick et al. 2018; Singh et al. 2020; Trivedi et al. 2020). This is supported by the significant and positive correlations

($P < 0.05$; r = 0.47–0.76) found between cotton yield and the relative abundance of these taxa in CS.

Of CS-enriched fungal biomarkers, members of Glomeraceae and Acaulosporaceae, known as AMF, are obligatory biotrophic organisms that form a mutualistic symbiosis with most crop plants. It is estimated that AMF provide up to 90% of P and 20% of N for their plant hosts through hyphal networks in exchange for fixed C from the host, increasing plant productivity (Smith and Read 2010). Glomeraceae was also found to serve as a core microbiome, with a substantially greater number of nodes and edges in CS-specific relative to ConR-specific fungal networks (Table S6). This suggests that the presence of Glomeraceae in CS may enhance the network connectivity and the complexity of microbial communities inside the cotton root, highlighting the significance of AMF for root microbiomes in CS. The relative abundance of Serendipitaceae, another core fungal microbiome in our study, was also positively correlated to cotton yield in CS ($P < 0.05$; r = 0.50), which could be due to its dual lifestyle: root endophytes or mycorrhizal fungi (Weiß et al. 2016). Serendipitaceae spp. can establish a mutualistic symbiosis with some crops, including maize, cotton, switchgrass, barley, and wheat, and could supply nutrients and water to the host crop (Yadav et al. 2010; Vohník et al. 2016; Ray et al. 2020). This effect was particularly significant in nutrient-limiting soils (Ray et al. 2020), consistent with the greater relative abundance of Serendipitaceae found in C2, which exhibited lower nutrient content compared to CS (Katsvairo et al. 2007c). However, our Pearson correlation analysis indicates that Serendipitaceae may contribute more to cotton growth in CS, although it showed lower relative abundance in CS, suggesting that the contribution of Serendipitaceae to cotton yield could be independent of their relative abundance in the root. This could be linked to functional redundancy within mycorrhizal fungal communities of ConR systems, as similar functions (e.g., nutrient supply to the hosts) were provided by mycorrhizal fungi (e.g., Glomeraceae, Gigasporaceae, Acaulosporaceae, and Serendipitaceae). Others demonstrated that microbial functional redundancy may be promoted by limitation in different trace nutrients (Louca et al. 2018). ConR systems, especially C2, had nutrient limitations in the past at this site (Katsvairo et al. 2007a; Zhao et al. 2010), although the extent of microbial functional redundancy in ConR systems, especially beneficial microbial communities, would need to be verified in future studies.

In contrast, core microbiomes exerted minor effects on plant growth promotion in ConR systems, with only one positive association between the relative abundance of Rhizobiaceae and cotton seed in C1. Instead, cotton yield in C2 was positively correlated with rotation-specific biomarkers (Polyangiaceae and Acidobacteriaceae; $P < 0.05$; r = 0.50–0.63; Fig. S5), suggesting that the benefits of divergent bacterial taxa on cotton productivity in SBR and ConR systems were highly variable. Overall, root microbiomes in CS may develop their ability to adapt to specific ecological niches through endophytic colonization of host crops, establishing an intimate association with benefits or no apparent harm to the host (Fitzpatrick et al. 2018). This is supported by the contribution of rotation systems to cotton productivity, via the assembly of core microbiomes in the cotton root (indirect coefficient = 0.11).

The linkage between rotation-specific networks and crop productivity

Previous studies have demonstrated that agricultural practices may alter the composition of root microbiome networks (Hartman et al. 2018; Banerjee et al. 2019), yet how these altered networks affect crop performance and productivity is still understudied. Here, we provide a holistic view of the associations of agricultural management, the composition of root microbiomes, and crop productivity over a 3-year period. CS-specific microbial networks yielded higher network complexity of bacterial and fungal communities, which can lead to significant plant-growth promotion based on the complementary roles of different taxa (Fig. 7). This is consistent with Durán et al. (2018) who reported that the network complexity of root microbiomes is highly linked to plant growth. Our study shows that CS led to profoundly greater modularity of bacterial and fungal networks relative to ConR systems, suggesting that CS induced complex ecological processes and stabilized microbial networks in root microbiomes, which could enhance root microbial functions and thereby promote cotton growth via a "plant legacy effect" (Ma et al. 2020; Hernandez et al. 2021). For example, the lower relative abundance of potentially pathogenic microbes (e.g., some taxa within the families of Sphingomonadaceae, and Muyocopronaceae) in CS indicates that the SBR system may restrict the assembly of pathogenic communities and the diseases they cause (Glaeser et al. 2014; Hernández-Restrepo et al. 2019). Previous research at this site also demonstrated that crop nutrient uptake (e.g., nitrogen, phosphorus, and potassium) was greater in the SBR system (Zhao et al. 2010). Overall, our results suggest that the greater temporal plant diversity in SBR promoted plant growth by enhancing the complexity and stability of root microbial networks (primarily root fungal network) associated with complex ecological processes (indirect coefficient = 0.39).

Conclusions

Our study investigated the impact of irrigation, rotation, and interannual variability on microbiome assembly in cotton roots. The responses of root microbial communities to these factors highlighted interannual variability as an important

driver of microbiome assembly in cotton roots. Compared to ConR, the greater plant diversity and trait variations in SBR enhanced root fungal diversity and microbial community complexity, leading to greater stability relative to fluctuating environmental conditions (e.g., temperature and precipitation) observed among sampling years. SBR had more beneficial microbiomes (e.g., Opitutaceae, Pseudonocardiaceae, Rhizobiaceae, Bacillaceae, Comamonadaceae, Serendipitaceae, and Glomeraceae) while restricting pathogens, implying that this crop rotation integrating 2 years of bahiagrass may preferentially select beneficial microbes to constitute core microbiomes, resulting in more mutually beneficial interactions with the cotton host. Root microbiomes in SBR fostered more complex and stable networks associated with complex ecological processes, contributing to a significant plant-growth promotion. Overall, these findings improve our understanding of the microbial mechanisms underlying the agronomic, environmental, and economic benefits observed when integrating perennial grasses in the conventional peanut-cotton rotation. Thus, future crop management that increases rotational diversity by integrating perennial grasses into row crop systems could select and harness beneficial microbes to sustainably promote agricultural productivity due to plant legacy effects (Hannula et al. 2019, 2020; Haskett et al. 2020). Additionally, future research studying plant microbiomes should be improved by directly eliminating plant DNA before sequencing (Aliche et al. 2021) and by sampling at least twice in a given year as changes in microbial diversity and community composition are often greater between different seasons than between different treatments.

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Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication All authors have approved the manuscript in its entirety and agreed for its publication.

Conflict of interest The authors declare no competing interests.

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