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Author/s:

Sun, A;Jiao, XY;Chen, Q;Trivedi, P;Li, Z;Li, F;Zheng, Y;Lin, Y;Hu, HW;He, JZ

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Title page

Fertilization alters protistan consumers and parasites in crop-associated microbiomes

Anqi Sun¹, Xiao-Yan Jiao², Qinglin Chen³, Pankaj Trivedi⁴, Zixin Li¹, Fangfang Li¹, Yong Zheng¹, Yongxin Lin¹, Hang-Wei Hu^{1,3,*}, Ji-Zheng He^{1,3}

1 Key Laboratory for Humid Subtropical Eco-geographical Processes of the Ministry of Education, School of Geographical Sciences, Fujian Normal University, Fuzhou 350007, China

2 College of Resource and Environment, Shanxi Agricultural University, Taiyuan 030031, China

3 School of Agriculture and Food, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, Victoria 3010, Australia

4 Microbiome Network and Department of Agricultural Biology, Colorado State University, Fort Collins, CO, USA

*For correspondence:

Hang-Wei Hu, Email: hangwei.hu@gmail.com;

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Originality-Significance Statement

We provide new insights into the diversity and functional traits of protists, a critical component of microbiota, in various plant-soil compartments (i.e. leaf phyllosphere, rhizosphere soil and bulk soil) in response to multiple fertilization regimes. Our results suggest that fertilization significantly influenced specific groups of protistan consumers and parasites, which may in turn alter the compositions of crop-associated bacterial and fungal communities from the top-down control in food webs. Our study advances the understanding of protists and their food web interactions in crop-associated microbiomes under intensive agricultural management practices, and highlights the potential of utilizing protists to engineer complex crop microbiomes with predictable outcomes.

Summary

Crop plants carry an enormous diversity of microbiota that provide massive benefits to hosts. Protists, as the main microbial consumers and a pivotal driver of biogeochemical cycling processes, remain largely understudied in the plant microbiome. Here, we characterized the diversity and composition of protists in sorghum leaf phyllosphere, and rhizosphere and bulk soils, collected from an eight-year field experiment with multiple fertilization regimes. Phyllosphere was an important habitat for protists, dominated by Rhizaria, Alveolata, and Amoebozoa. Rhizosphere and bulk soils had a significantly higher diversity of protists than the phyllosphere, and the protistan community structure significantly differed among the three plant-soil compartments. Fertilization significantly altered specific functional groups of protistan consumers and parasites. Variation partitioning models revealed that soil properties, bacteria and fungi predicted a significant proportion of the variation in the protistan communities. Changes in protists may in turn significantly alter the compositions of bacterial and fungal communities from the top-down control in food webs. Altogether, we provide novel evidence that fertilization significantly affects the functional groups of protistan consumers and parasites in crop-associated microbiomes, which have implications for the potential changes in their ecological functions under intensive agricultural managements.

Introduction

Protists are enormously diverse and abundant in soil (Adl and Coleman, 2005), and are a pivotal component of soil food webs (Geisen et al., 2018; Nguyen et al., 2020; Oliverio et al., 2020). Soil protists, accounting for a significant proportion of soil eukaryotes (de Araujo et al., 2018), are recognized as a key driver of biogeochemical nutrient cycling processes (Geisen et al., 2017), and a major contributor to energy turnover and transfer across trophic levels of soil food webs (Oliverio et al., 2020). More importantly, protist predation of microorganisms as food sources is considered as a crucial factor modulating the dynamics of belowground microbial eukaryotes and prokaryotes, with consequences for a wide range of ecosystem functions (Ishii and Shimada, 2012; Thakur and Geisen, 2019; Karakoç et al., 2020). Recent studies have reported the role of protists in plant disease control and plant growth promotion, possibly through ingestion of plant pathogenic bacteria (Xiong et al., 2020) or stimulating plant growth promoting bacteria (Jousset, 2012). Despite being functionally important, however, the diversity and function of protists, and their interactions with other microorganisms, in the plant-soil systems have received less attention. It is fundamental to disentangle the role of protists in soil ecological processes and agricultural productivity, which can provide new insights into engineering microbiomes for sustainable agriculture and food security.

Plants are a vastly complex biological system and provide multiple habitats for the growth and proliferation of an immense diversity of microbiota (i.e. the plant microbiome), including bacteria, fungi, archaea, protists, and viruses (Trivedi et al., 2020). Intimate relationships between plants and microorganisms have been recognized as a key driving force that impacts nutrient acquisition, immune development, and pathogen resistance (Sessitsch and Mitter, 2015; Trivedi et al., 2020), which is critical in regulating plant health and productivity (Liu and Brettell, 2019; Qiu et al., 2019). Recent omics-based identification and characterization of microbiomes has greatly expanded the repertoire of microorganisms associated with plants and their surrounding environment (Trivedi et al., 2020). The majority of these studies, however, have focused merely on the bacterial and fungal communities (Edwards et al., 2015; Müller et al., 2016; Sun et al., 2021), but largely ignored the assembly of plant-associated protists and their relationships with other microbial communities (Sapp et al., 2018). A full appreciation of the mechanisms that govern the distribution and abundance

of plant-associated protists, is a prerequisite to unlock their potential to promote agricultural productivity and sustainability (Qiu et al., 2019).

The assembly and stability of crop microbiomes are jointly influenced by a wide range of abiotic and biotic factors (Trivedi et al., 2020). There is an increasing recognition that common agricultural management practices, such as organic and inorganic fertilizations, have significant effects on the dynamics, structure and functions of plant and soil microbiomes over time (Bonanomi et al., 2018; Schmidt et al., 2019; Zhao et al., 2020). Soil protists are known to respond to changes in soil physicochemical conditions such as soil pore size, moisture content, pH, and nutrients (Oliverio et al., 2020; Zhao et al., 2020). Application of fertilizers, therefore, would impact the plant-associated protistan communities through directly modifying soil properties, or indirectly altering the communities of bacteria and fungi (Allison et al., 2007; Álvarez-Martín et al., 2016) which can impact protists through the trophic food web interactions (Geisen et al., 2018). Plants are made of distinct tissues and organs that provide different habitats for microbiota, which may differentially respond to agricultural management practices across different compartments. As protists are recognized as key regulators of microbiome assembly (Guo et al., 2019), changes in the diversity and composition of protists in response to fertilization can have significant effects on community assembly and stability, with unknown consequences for the key functions performed by the crop microbiomes.

In this study, we evaluated the effects of agricultural management practices (including inorganic, organic and mixed fertilization) on the protistan communities, and their relationships with the bacterial and fungal communities, in various compartments of sorghum plant (leaf phyllosphere, rhizosphere soil and bulk soil) based on an eight-year field experiment. Sorghum is the fifth most produced cereal grain in the world and has strong resistance to barren and drought (Gao et al., 2020). The ability of plants to face environmental stresses is partly mitigated by its associated microbiome. However, the diversity and composition of microbiota, especially protists, associated with sorghum plants remain largely unknown. Here, we characterized protists, bacteria, and fungi in various plant-soil compartments through amplicon sequencing of 18S rRNA gene, 16S rRNA gene and internal transcribed spacer (ITS) region, respectively. We tested the hypotheses that (i)

fertilization would influence the functional groups of protists, through altering soil properties and the bacterial and fungal communities, the main food sources of protists; and (ii) changes in protists would in turn alter the compositions of bacterial and fungal communities from the top-down control in food webs.

Results

Compositions of eukaryotic communities across different plant-soil compartments

We analyzed differences in the diversity and composition of eukaryotic communities among different compartments of sorghum (including leaf phyllosphere and rhizosphere soils) and bulk soils collected from the agricultural field with multiple fertilization regimes. Amplicon sequencing of the 18S rRNA gene resulted in a total of 2,219,616 eukaryotic sequences across all soil and plant samples. Protists were the most dominant eukaryote (33.88% of the total eukaryotic sequences) in both rhizosphere and bulk soils, compared to 12.28% in the phyllosphere (Fig. 1A). Rhizaria were the most abundant supergroup of protists across all compartments, followed by other supergroups including Alveolata, Amoebozoa, and Stramenopiles (Fig. 1A). Phyllosphere was dominated by three major supergroups including Rhizaria, Alveolata, and Amoebozoa (Fig. 1A). Four rare supergroups of protists were also detected: Opisthokonta, Hacrobia, Excavata and Apusozoa.

Changes in the protistan communities across different fertilization treatments

The Good's coverage of protists was $99.61\% \pm 0.27\%$, $98.61\% \pm 0.33\%$, and $98.47\% \pm 0.31\%$ in the phyllosphere, rhizosphere soil, and bulk soil, respectively (Fig. S1A), indicative of sufficient sequencing depth to describe the protistan community diversity. The Shannon diversity of protists was significantly lower in the phyllosphere than in rhizosphere and bulk soils ($P < 0.05$) (Fig. 1B). The eight-year fertilization treatments did not significantly change the Shannon diversity of protists in any sorghum-soil compartment ($P > 0.05$) (Fig. 2A). Community compositions of protists varied significantly among the compartments, as revealed by the distinct cluster of phyllosphere samples well separated from the cluster of rhizosphere and bulk soil samples (Fig. 1C). However, fertilization did not significantly alter the community compositions of protists in any sorghum-soil compartment ($P > 0.05$) (Fig. 2B). Source-tracker analysis revealed that rhizosphere and bulk soils accounted for 0.90% of the protistan taxa found in the phyllosphere (Fig. 1D).

Our further analyses of protists at the class level revealed that Spirotrichea and Colpodea in the phyllosphere were significantly influenced by the fertilization treatments (Fig. S2). Fertilization significantly enhanced the relative abundances of Phyllopharyngea, Oligohymenophorea, and Lobosa_X, but decreased the relative abundance of Hyphochytriomyceta in rhizosphere soils; Filosa-Sarcomonadea, Endomyxa-Phytomyxea, Filosa-Imbricatea, Oligohymenophorea, Phyllopharyngea and Spirotrichea, and Tubulinea were enriched, while Bacillariophyta, Chrysophyceae, and Hyphochytriomyceta were decreased by fertilization in bulk soils (Fig. S2).

Changes in the functional groups of protists across different fertilization treatments

Functional groups of protists were dominated by consumers, parasites, phototrophs, and Undetermined (Fig. S3). The relative abundances of total consumers, parasites, and phototrophs remained largely unchanged across the fertilization treatments, except parasites in rhizosphere soils (Fig. S3B) as well as parasites and phototrophs in bulk soils (Fig. S3C). In the phyllosphere, Amoebozoa belonging to consumers was significantly influenced by fertilization (Fig. 3A). In rhizosphere soils, the relative abundances of Apicomplexa_X and Oomycota belonging to parasites were significantly enhanced by fertilization (Fig. 3B). In bulk soils, Apicomplexa_X and Oomycota belonging to parasites, as well as Litostomatea, Tubulinea, and Variosea belonging to consumers, were significantly influenced by fertilization (Fig. 3C).

We further performed a functional trait-based classification of the Cercozoa phylum according to their prey preferences (Fig. S4). Cercozoa accounted for 45.8%, 21.7%, and 30.9% of the total protistan consumers in the phyllosphere, rhizosphere soil and bulk soil, respectively. The phyllosphere Cercozoa was dominated by bacterivores (73.8%), while bacterivores, omnivores and parasites were all highly abundant in rhizosphere and bulk soils (Fig. S4A). The relative abundances of the cercozoan functional groups in the phyllosphere and rhizosphere soil showed no significant response to fertilization, but bacterivores, omnivores and parasites in bulk soil significantly changed in specific fertilization treatments (Fig. S4B).

Overview of the bacterial and fungal communities in different sorghum-soil compartments

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Amplicon sequencing of the bacterial 16S rRNA gene and fungal ITS region yielded a total of 1,624,266 and 2,682,342 reads, respectively. The Good's coverage was $99.75\% \pm 0.06\%$, $98.74\% \pm 0.06\%$, and $95.79\% \pm 0.31\%$ for bacteria, compared to $99.82\% \pm 0.02\%$, $98.72\% \pm 0.003\%$, and $95.74\% \pm 0.03\%$ for fungi, in the phyllosphere, rhizosphere soil, and bulk soil, respectively (Fig. S1A), indicating that sufficient sequencing depth was achieved to describe the bacterial and fungal community diversity. The observed OTU richness and Shannon diversity of both bacteria and fungi were significantly higher in rhizosphere and bulk soils than those in the phyllosphere (Fig. S1B and S1C).

Drivers of the protistan communities in different sorghum-soil compartments

We conducted variation partitioning models to identify the effects of soil properties, bacterial communities, and fungal communities in driving the changes of the protistan community compositions. Soil properties, bacterial communities, and fungal communities together explained 63%, 38%, and 71% of the total variation in protistan communities in phyllosphere, rhizosphere soil, and bulk soil, respectively (Fig. 4). The interactions of soil properties, bacterial communities, and fungal communities were the most dominant factor predicting the changes of protists in all the sorghum-soil compartments (20% for the phyllosphere, 11% for rhizosphere soil, and 18% for bulk soil). Bacteria was the most important predictor of protists in the phyllosphere and rhizosphere soil, while soil properties were the most important predictor in bulk soil (Fig. 4). Overall, microbial factors including both bacteria and fungi could explain a larger proportion of the changes in protists than soil properties for all the compartments.

The strong correlations of protistan, bacterial and fungal communities were further corroborated by the many significantly relationships between protists vs. bacteria, and protists vs. fungi in all the sorghum-soil compartments (Figs. S5 and S6). The Shannon diversity of protists was significantly and positively correlated with both bacterial and fungal community diversity (Fig. S7). The co-occurrence network including protistan, bacterial and fungal groups suggested that protists formed distinct hubs linking a broad diversity of bacterial and fungal taxa in the network (Fig. 5). In particular, taxa belonging to the protistan phyla including Cercozoa, Chlorophyta, Conosa, Lobosa, Ochrophyta, and Streptophyta_X were intensively associated with taxa of the dominant bacterial and fungal phyla, with

majority of the associations being positive. The bacterivores and omnivores of the Cercozoa phylum had positively interactions with bacterial taxa but not fungal taxa. Bacterial taxa also had intensive (both positive and negative) interactions with fungal taxa, indicating their shared ecological niches or competitive relationships in agricultural soils under fertilization treatments.

We used sorghum yield per acre and protein content as indexes of the productivity and quality of sorghum, respectively. Random forest regression modelling was conducted to identify the protistan taxa at the genus level best predicting sorghum yield and protein content across the fertilization treatments (Fig. S8). The protistan genera were incorporated as the main biological predictors in our models, and the top genera that best predicted sorghum yield and protein content are shown. We found that *Platyophrya*, *Leptopharynx* and *Colpodidae_X* in the phyllosphere, *Spumella*, *Aplanochytrium* and *Hyphochytrium*, *Aplanochytrium* in rhizosphere and bulk soils were the dominant predictors of both sorghum yield (Fig. S8A) and protein content (Fig. S8B).

We further constructed a structural equation model (SEM) to explore the casual relationships among fertilization, sorghum-soil compartments, soil properties, and protistan, bacterial and fungal community compositions (Fig. 6A). Sorghum-soil compartment was the most dominant driving force shaping the pattern of protistan and fungal community compositions, while protists were the most important predictor of the bacterial community composition (Fig. 6B). The protistan community could also significantly impact the bacterial and fungal community compositions. Soil properties and fertilization were important predictors had only marginal effects on the bacterial, fungal and protistan community compositions (Fig. 6B).

Discussion

The spatial organization of the crop microbiome influences various crop properties including colonization, metabolism, plant-microbe interactions and crop performance (Fitzpatrick et al., 2020). Managing the crop microbiome remains a major challenge, mainly due to the daunting complexity of microbiota residing in various plant-soil compartments. In agriculture, we are just beginning to explore opportunities to manage the complexity of crop microbiomes (Müller et al., 2016) and develop microbiome-based products in farming

practices (Toju et al., 2018). However, protists, as a critical component of microbiota that are essential to community stability, are generally missing from the majority of previous crop microbiome studies (Sapp et al., 2018). New insights into this important microbial group will improve our knowledge of the ecological processes that govern crop microbiome assembly and will contribute to practical measures to improve crop fitness and agricultural productivity.

We found clear differences among the protistan communities in different sorghum-soil compartments (phyllosphere, rhizosphere and bulk soils) (Fig. 1), suggesting that the plant-soil compartment is a strong selection force shaping the composition of protists, even under the disturbance of fertilization. This finding is consistent with previous reports that different plant-soil compartments (e.g. leaves, roots, or flowers) typically harbour unique microbiota, though protists were not included in these studies (Gómez and Ashman, 2019; Leveau, 2019; Massoni et al., 2020; Pascale et al., 2020), except that a clear distinction of Cercozoa (Rhizaria) and Oomycota (Stramenopiles) was found between compartments of the model plant *Arabidopsis thaliana* (Sapp et al., 2018). Our results showed that protists in sorghum-associated microbiomes were predominated by the supergroup Rhizaria. Fine tuning between plant immune response and microbe genomic traits are known to regulate endophytic colonization (Trivedi et al., 2020). Interestingly, the cell-surface G-protein-coupled receptors (GPCR) mediated signalling pathway are significantly enriched in the Rhizaria supergroup (Bi et al., 2019). GPCRs sense a diverse array of extracellular signals from plant host and are reported to promote endophytic colonization (Xu et al. 2014). Further research is required to elucidate molecular plant-protist interactions that result in the selective recruitment of few functional groups in the endosphere. As for protistan functional groups, although all the four compartments are dominated by consumers, we found that (i) phototrophs are significantly more abundant in the phyllosphere; and (ii) parasites are an important functional group of protists in rhizosphere and bulk soils (Fig. S3). Protistan phototrophs can obtain energy via photosynthesis, therefore, it is not surprising to detect them in leaf phyllosphere, which indicates their potential involvement in photosynthesis by using the plant phyllosphere as the habitat. Protistan parasites can parasitize on various hosts (animals, plants, or other organisms), and rhizosphere and bulk soils provide the most abundant sources of host for

them, which may explain their presence in these soil habitats. Protistan consumers (or predators) prey on a wide range of bacteria and fungi as well as other eukaryotes (Hiltunen et al., 2012; Geisen et al., 2018). Their dominance may imply the potential role in regulating the bacterial and fungal communities in the crop microbiome, affecting ecosystem-level processes such as nutrient cycling and pathogen control.

We found that the diversity of protists declined from rhizosphere and bulk soil to leaf (Fig. 1B), indicating the increasing host-specific selective habitat filtering and recruitment of protists at the root-soil and leaf-air interface (Wagner et al., 2016; Trivedi et al., 2020). Plants possess epithelial surfaces where the host releases a multitude of nutrients, antimicrobials, organic acids, and mucus or mucilage, which are thought to shape the crop microbiome (Foster et al., 2017). Source-tracker analysis revealed that rhizosphere and bulk soils accounted for only 0.90% of phyllosphere protists (Fig. 1D). Plant microbiome assembly models suggest that specific microbes in the environment colonize plant surfaces, followed by additional filtering as microbial taxa are recruited into the interior of plant organs (Bulgarelli et al., 2013). Leaf phyllosphere is an open environment, and surface colonizing protists may disperse from the surrounding environment, such as soil, neighboring plants, insects, animals and aerosols, or migrate from other plant tissues (Gómez and Ashman, 2019), though plant endophytes were not characterized in the current study. Plant genetic traits that can mediate leaf tissue chemistry and the surface topology (Liu et al., 2020) may also contribute to the colonization of phyllosphere protists.

Fertilization did not significantly influence the diversity and composition of total protists (Fig. 2), suggesting that protists have a high tolerance to fertilization disturbance, but a few functional groups of protistan consumers and parasites are more responsive to fertilization than other functional groups (Fig. S2). Within the trophic functional groups of protists, we found that the relative abundances of some lineages of protistan consumers and parasites were substantially influenced by fertilization (Fig. 3), and bacterivores, omnivores and parasites of the Cercozoa phylum in bulk soil were significantly responsive to fertilization (Fig. S4). The changes in protistan consumers and parasites could be due to changes in (i) soil properties or (ii) the bacterial and fungal communities, which are main preys (i.e. food sources) for protists (Fig. 4). Fertilization provides nutrients for the plant and

the growth of bacteria and fungi, which serve as food sources to maintain and facilitate the growth of specific protistan consumers (Fig. 3). Fertilization can also harm soil bacterial group like Planctomycetes (Eo and Park, 2016), which in turn can affect the protist community through the bottom-up pathway. Therefore, it is not surprising to observe the increase in some protistan taxa of consumers under fertilization. Many bacteria can defend themselves against certain protist predators and even kill them (Geisen et al., 2017), and thus the promotion of these bacteria by fertilization may result in decline of corresponding protistan consumers (Fig. 3). For parasites, the changes of this trophic group by fertilization may be explained by that fertilization may also influence main hosts (metazoan, plants and other soil organisms) of parasitic protists (Schulz et al., 2019), with indirect effects on parasites. Together, our results highlight that specific functional groups of consumers and parasites are susceptible to fertilization in the crop microbiome.

Ecological theories suggest that the plant-associated microbial community assembly is governed by complex interactions among microorganisms, their plant host and the environment (Trivedi et al., 2020). Biotic food web interactions (e.g., top-down and bottom-up processes) among microbes at multiple trophic levels are crucial for ecosystem functioning and can also alter the crop microbiome via both antagonistic and beneficial interactions (Schulz-Bohm et al., 2017; Durán et al., 2018; Carrión et al., 2019; Uroz et al., 2019). Protistan consumers can function as bacterivores, eukaryvores, and omnivores (Geisen et al., 2018), and the bacterivore and omnivore of the Cercozoa phylum could be significantly influenced by fertilization in bulk soil (Fig. S4). Protistan consumers such as Cercozoa could differentially regulate bacterial communities via grazing as reported previously for the genera Allapsa, Cercomonas, Paracercomonas, and Sandona (Glücksman et al., 2010). The grazing pressure of bacterivorous protists significantly impact the composition and function of plant-associated bacteria (Rosenberg et al., 2009; Flues et al., 2017). Changes in the consumers ultimately can also stimulate the activity of other organisms (de Vries et al., 2020), and alter the flow of nutrients through food webs and can affect ecosystem processes such as nutrient cycling. At the same time, bacteria and fungi, as the key food sources of protists, may in turn shape the assembly process of the plant-associated protists. In this study, our SEM results revealed that the protistan community could significantly impact the bacterial and fungal

community compositions through the top-down control (Fig. 6), and the compositions of these microbial groups were intensively and mostly positively correlated (Fig. 5), suggesting bacteria and fungi can be important food sources impacting the growth of protists via bottom-up regulations. Although we cannot provide direct evidence for the predation of bacteria by protists, the network analysis revealed intensive positive correlations between bacterivore and the bacterial communities (Fig. 5), suggesting specific prey preferences of these protists and their role in modulating the plant microbiome should be fully explored in future studies. Previous studies have shown that (i) feeding by protists modified the community composition of bacteria, resulting in functional changes in the bacterial community structure (Schulz-Bohm et al., 2017); and (ii) predators can boost causal interactions among community members and lead to higher coexistence among prey species (Karakoç et al., 2020). Therefore, changes in these functional groups of protists by long-term intensive management practices would ultimately affect the assemblages of plant-associated microbiomes. These findings have fundamental ecological implications for future efforts to promote beneficial food web interactions or harness the potential of protists as bacterial biocontrol agents to manipulate nutrient cycling and maximize crop resilience to biotic/abiotic stresses.

It should be noted that the primer pair ITS1F/ITS2R (White et al., 1990; Gardes and Bruns, 1993) used to detect potential prey fungi is not up to date, as it may heavily amplify plant sequences and has been superseded by primers with better coverage (Toju et al., 2012; Bokulich and Mills, 2013). This drawback in primer selection may affect the linkages between protists and the fungal taxa that may have been missed due to primer bias. Although the primers to target protists were properly chosen, some important plant associated protist groups such as Cercozoa (Fiore-Donno et al., 2017) and Oomycetes (Sapp et al., 2019), were still missed to some extent using general primer sets. This issue may be resolved by using V4 of 18S rRNA gene metabarcoding with newly designed primers (Fiore-Donno et al., 2018) to cover nearly the total diversity of Cercozoa and the Internal Transcribed Spacer 1 (ITS1) to determine oomycetes, as reported in Sapp et al. (2018).

Conclusions

In conclusion, our study provides novel insights that fertilization significantly altered the relative abundances of specific protistan consumers and parasites in sorghum-associated

compartments (phyllosphere, rhizosphere soils and bulk soils). Fertilization-induced changes in the protistan communities could be attributed to the interactions of bacterial/fungal communities and soil properties, and in turn, protists may exert top-down control on the communities of bacteria and fungi, with unknown consequences for their regulated ecosystem functions and crop performance. Our study highlights the necessity to consider protist and their interactions with other microbes in evaluating the effects of agricultural management practices on crop associated microbiomes. Understanding the dynamics of protists and their food web interactions will provide a way forward to engineer complex crop microbiomes with predictable behaviour and robust outcomes. Future phytobiome research should account for the various components within the plant habitat, especially when insights should be applied for plant health.

Experimental procedures

Site description and soil sampling

The experimental site was located at the Dongyang long-term experimental station (37°33'21"N, 112°40'2"E) in Shanxi Academy of Agricultural Sciences, Shanxi Province, China. This region has a mean annual temperature and precipitation of 9.7°C and 321 mm, respectively. The soil is classified as typical carbonate cinnamon soil with a sandy loam texture (1.7% clay, 11.6% silt, and 86.7% sand). The fertilization experiment was established in 2011 with a cropping system of sorghum-maize rotation, aiming to understand the effects of different fertilization regimes on the soil fertility and productivity. The experiment consisted of six treatments in a randomized block design (15 × 5 m² for each plot) of three replicates: no fertilizer (CON), mineral nitrogen (N), phosphorous (P) and potassium (K) combination (NPK), organic manure plus straw application (MS), mineral fertilizers NPK plus organic manure (NPKM), mineral fertilizers NPK plus organic manure and straw application (NPKMS), and mineral fertilizers plus organic manure and straw application by deep ploughing (NPKMSD). In the NPK treatment, N, P and K were applied as urea (225 kg N hm⁻² per year), diammonium phosphate (60 kg P₂O₅ hm⁻² per year), and potassium sulphate (60 kg K₂O hm⁻² per year). For the MS, NPKM, NPKMSD and NPKMS treatments, organic manure (i.e. cow dung) was applied at the same rate of 45 m³ hm⁻², equivalent to 100 kg N hm⁻², 56 kg P₂O₅ hm⁻² and 69 kg K₂O hm⁻². For all straw application treatments (MS,

NPKMS, and NPKMS), 6150 kg straw hm^{-2} was applied, equivalent to 49 kg N hm^{-2} , 11 kg P_2O_5 hm^{-2} and 139 kg K_2O hm^{-2} . The deep ploughing treatment was applied in 0-30 cm layers while the others were in 0-20 cm. All organic manure, straw and PK were amended as basal fertilization, while half of N was amended as basal fertilization, and another half as topdressing at the jointing stage of sorghum growth.

Soil and plant samples were collected at the ripening stage of sorghum in September 2019. Bulk soils were collected from a distance of 30 cm away from the plants within each plot by mixing five random soil cores (0-10 cm). Sorghum leaves were collected at the same height of sorghum plants (~60 cm above soil surface) using sterilized scissors and packed into clean sterilized bags. Sorghum plants were removed from the ground, and soils attached to the surface of sorghum roots were regarded as rhizosphere soils. All samples were transported to the laboratory in an ice box. Soil samples were sieved < 2-mm, stored at 4°C for three days prior to analyses of soil physicochemical properties and subsamples were frozen immediately at -80 °C for molecular analyses. The sorghum leaf samples were immediately treated for DNA extraction upon arrival.

Measurement of soil physicochemical properties

Soil properties were determined following conventional methods. Briefly, soil dissolved organic carbon (DOC) was analyzed in water extracts (1:4 of the soil : water ratio) for 0.4 h after filtration through 0.45 μm filter (Matlou and Haynes, 2006). Soil total nitrogen (TN) and total carbon (TC) were determined by the combustion method on an element analyzer using air-dried soils (Vario MAX C/N, Germany). Soil mineral N (NO_3^- -N and NH_4^+ -N) concentrations were analyzed using a flow analyzer (Skalar, Holland) (Wyngaard et al., 2018). Soil total phosphorus (TP) was extracted using a heating-acid-digestion method and determined with a flow analyzer (Pierzynski, 2000).

Genomic DNA extraction

Soil genomic DNA was extracted using the DNeasy PowerSoil DNA Isolation kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. For collecting phyllosphere microorganisms, about 5.0 g leaf samples were added into a conical flask with 100 ml phosphate buffer saline, and shaken at 180 rpm at room temperature in an incubator for 1 h after ultrasonic shaking for 5 min. The washing solution was passed through sterilized

medical gauze and a 0.22- μ m filter by Vacuum Filtration System (MultiVac610-MS-T, Rocker, China). The DNA extraction of leaf phyllosphere samples was performed using the DNeasy PowerSoil DNA Isolation kit (Qiagen). The quality of DNA extracts was determined by the NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MI, USA).

Amplicon sequencing and bioinformatic analyses

We used the primer sets 515F/806R (Bates et al., 2011), ITS1F/ITS2R (White et al., 1990; Gardes and Bruns, 1993), and F-TAR-euk454FWD1/R-TAReukREV3 (Stoeck et al., 2010) to amplify the bacterial 16S rRNA gene, fungal ITS1 region and the eukaryotic 18S rRNA gene, respectively, from all soil and plant samples. PCR products were purified, pooled and sequenced on the Illumina Miseq platform (Majorbio, Shanghai, China). The raw sequences were discarded if they contain ambiguous nucleotides, have a low ($Q < 20$) quality score, and are short in length (< 100 bp) (Chen et al., 2020).

The high-quality sequences were analyzed using the standard operating procedure in QIIME (Caporaso et al., 2010). Protistan sequences based on the eukaryotic 18S rRNA gene data were taxonomically assigned against the Protist Ribosomal Reference (PR2) database (Guillou et al., 2012). Taxonomic identification of bacteria and fungi was obtained against the Silva (Peplies, 2007) and UNITE database (Nilsson et al., 2018), respectively. Bacterial sequences that match host mitochondria and chloroplast were removed. Operational taxonomic units (OTUs) at 97% similarity were identified using the UPARSE v7.0.1090 (Edgar, 2013). The resampling of 30,828 reads for protists, 30,079 reads for bacteria, and 47,541 reads for fungi was conducted to account for the difference in sequencing depth of different samples, using the 'sub.sample' command in Mothur (Schloss et al., 2009). Alpha diversity was calculated using the Shannon diversity index to compare the microbial diversity across different compartments and treatments.

The trophic functional groups of the protistan community, including consumers, parasites, phototrophs and undetermined, were assigned as previously described (Nguyen et al., 2020; Oliverio et al., 2020). We further classified the cercozoan OTUs into bacterivores, eukaryvores (predating fungi, algae, protists, and small animals), omnivores (predating both bacteria and eukaryotes) as previously described (Fiore-Donno et al., 2019). We have deposited all raw sequences in the NCBI Sequence Read Archive (SRA) under the accession

number PRJNA664548.

Statistical analyses

To determine the potential compositional differences in the protistan community across compartments and treatments, principal coordinates analysis (PCoA) was performed using the “vegan” package (Oksanen et al., 2012) in R (v3.6.2) based on the Bray-Curtis dissimilarity. Multifactorial permutational analysis of variance (PERMANOVA) was conducted using the “vegan” package (Oksanen et al., 2012) to test the differences in the protistan community compositions between different fertilization treatments and sorghum-soil compartments. The first component (PCoA1) of the PCoA analysis was used to represent the variation in protistan community compositions in the subsequent analyses. The significant differences in the protistan alpha diversity between different fertilization treatments were determined using analysis of variance (ANOVA) in SPSS 22 (IBM, USA) at $P < 0.05$. We used SourceTracker analysis to calculate the transmission of the protistan communities across the four plant-soil compartments as described previously (Gou et al., 2017). Variation partitioning was performed to quantify the relative contribution of soil properties, and bacterial and fungal community compositions, to the variation in the protistan community composition with the ‘varpart()’ function in the “vegan” package (Legendre, 2007). Visualization of the variation partitioning result was performed with the ‘upset()’ function in the “UpSetR” package. The response ratios were computed by the mean relative abundance of protists in the treatment and control group (Nguyen et al., 2020). To explore the co-occurrence patterns of protistan, bacterial and fungal taxa, network based on pairwise comparison was built. A Spearman’s correlation was considered statistically robust if the Spearman’s correlation coefficient (R) was > 0.8 or < -0.8 and the P -value was < 0.01 (Barberán et al., 2012). The Spearman’s correlation was calculated using “psych” package. The co-occurrence network was visualized using CYTOSCAPE 3.6.0 (Shannon et al., 2003).

Structural equation model (SEM) was used to analyze the relationships among fertilization treatments, soil properties (pH, DOC, TN, TC, C/N, TP, NO_3^- -N and NH_4^+ -N), sorghum-soil compartments, bacterial, fungal and protistan community compositions. PCoA1 was used to proxy the variance of bacterial, fungal and protistan community composition, respectively. The *a priori* models included all possible pathways among these factors. To

reduce the complexity of SEMs, the representing indices of soil properties were calculated by PCoA using the “vegan” package. The significance of each path-coefficient was analyzed by calculating its critical ratio ($P < 0.05$). The overall model fit was evaluated with the Bentler comparative fit index (CFI), goodness-of-fit index (GFI), and Chi-square test. The SEM was performed using Amos Graphics v22 (IBM Corp., Armonk, NY, USA).

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Figure captions

Figure 1 Compositions of eukaryotic communities across different sorghum-associated compartments. The right panel shows the relative abundances of protist supergroups (A). The Shannon diversity for the protistan communities across different sorghum-associated compartments (B). Different letters above the boxes indicate significant differences at $P < 0.05$. Principal coordinate analysis (PCoA) grouped by compartments based on the Bray-Curtis distance showing the overall distribution pattern of the protistan community (C). The source-tracker analysis showing the transmission of protists among the sorghum-associated compartments (D).

Figure 2 Changes in the Shannon diversity of the protistan communities across the fertilization treatments in the three sorghum-associated compartments (A). Principal coordinate analysis (PCoA) of the protistan communities based on the Bray-Curtis distances (B). No fertilizer (CON), mineral N, P and K combination (NPK), organic manure plus straw application (MS), mineral fertilizers plus organic manure (NPKM), mineral fertilizers plus organic manure and straw application by deep ploughing (NPKMSD), and mineral fertilizers plus organic manure and straw application (NPKMS). “NS” represents no significant differences.

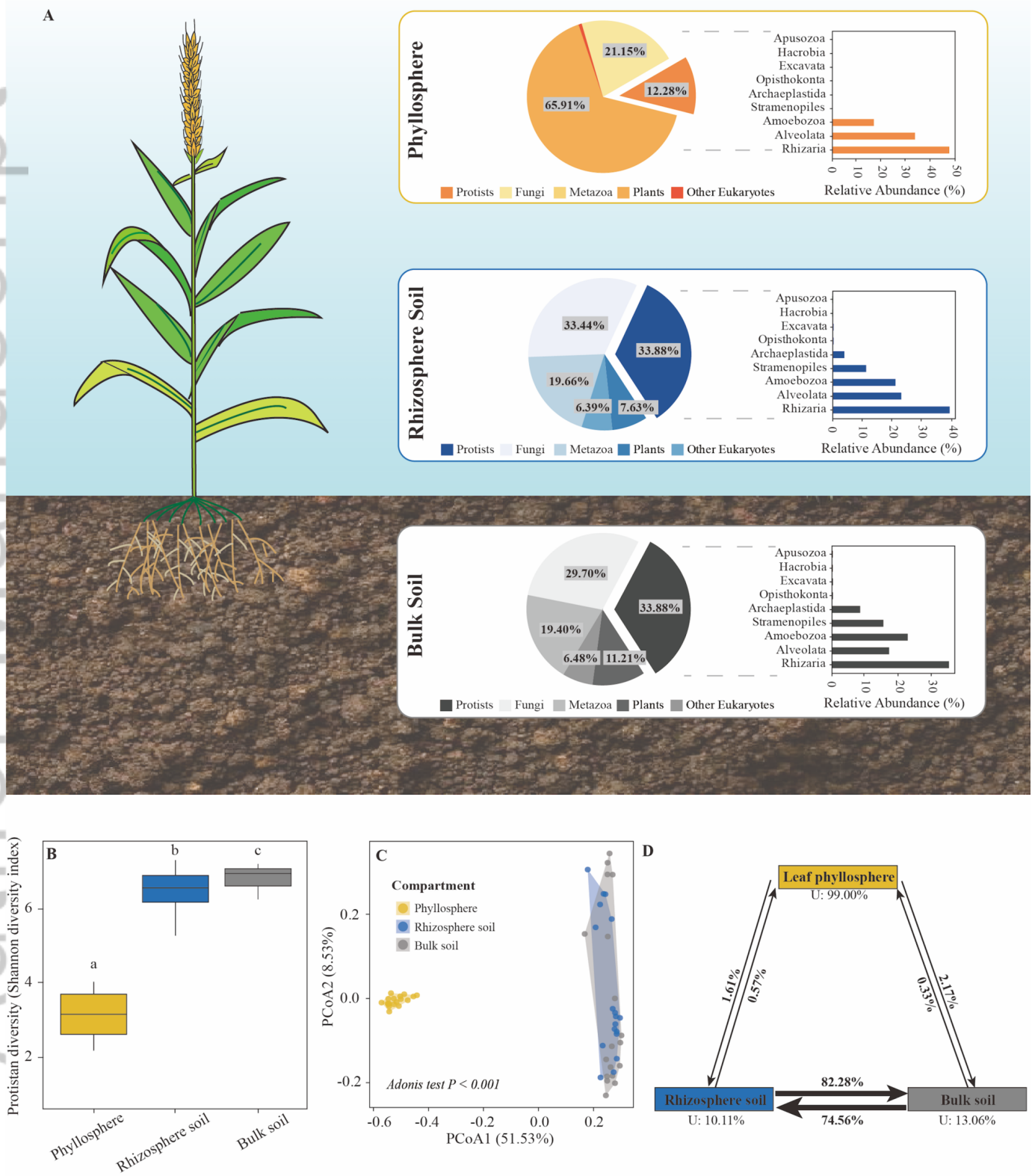
Figure 3 Response ratios of the relative abundances of functional protist lineages to the fertilization treatments. Only the protist lineages (at the class level) of consumer and parasite groups with significant differences compared to controls ($*P < 0.05$ and $**P < 0.01$, ANOVA) are plotted on a log base 2 scale. Error bars indicate standard errors ($n = 3$).

Figure 4 UpSet plots showing the effects of soil properties, bacterial communities, and fungal communities on the protistan communities in different sorghum-associated compartments, as revealed by variation partitioning models. Soil properties consisted of pH, DOC, TN, TC, C/N, TP, NO_3^- -N and NH_4^+ -N. The points in the matrix or the points connected by line represent the driving factors of corresponding bar. The total size of each driving factor is represented on the left barplot. Every possible intersection is represented by the bottom plot, and their occurrence (i.e., the numbers above bars) is shown on the top barplot.

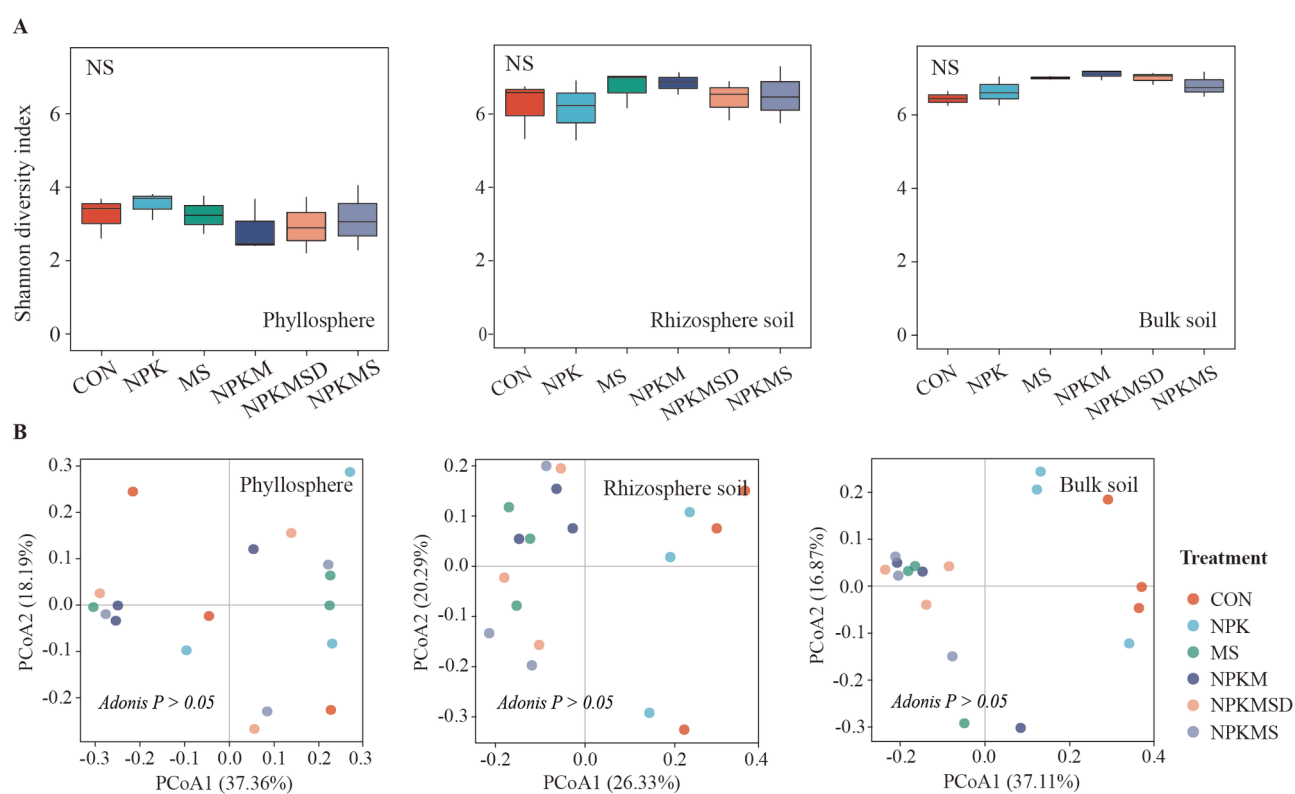
Figure 5 Co-occurrence network visualized by Cytoscape shows the associations among

bacterial, fungal and protistan taxa. The Cercozoa phylum are further classified into bacterivores and omnivores. A connection stands for a strong (Spearman's $|R| > 0.8$) and significant ($P < 0.01$) correlation. Red, green and blue nodes represent associated bacterial, fungal and protistan taxa, respectively. The size of each node is proportional to the number of connections. The thickness of each edge is the value of Spearman correlation coefficient. The green lines show the positive relationships and the red lines show the negative relationships between the bacterial, fungal and protistan communities.

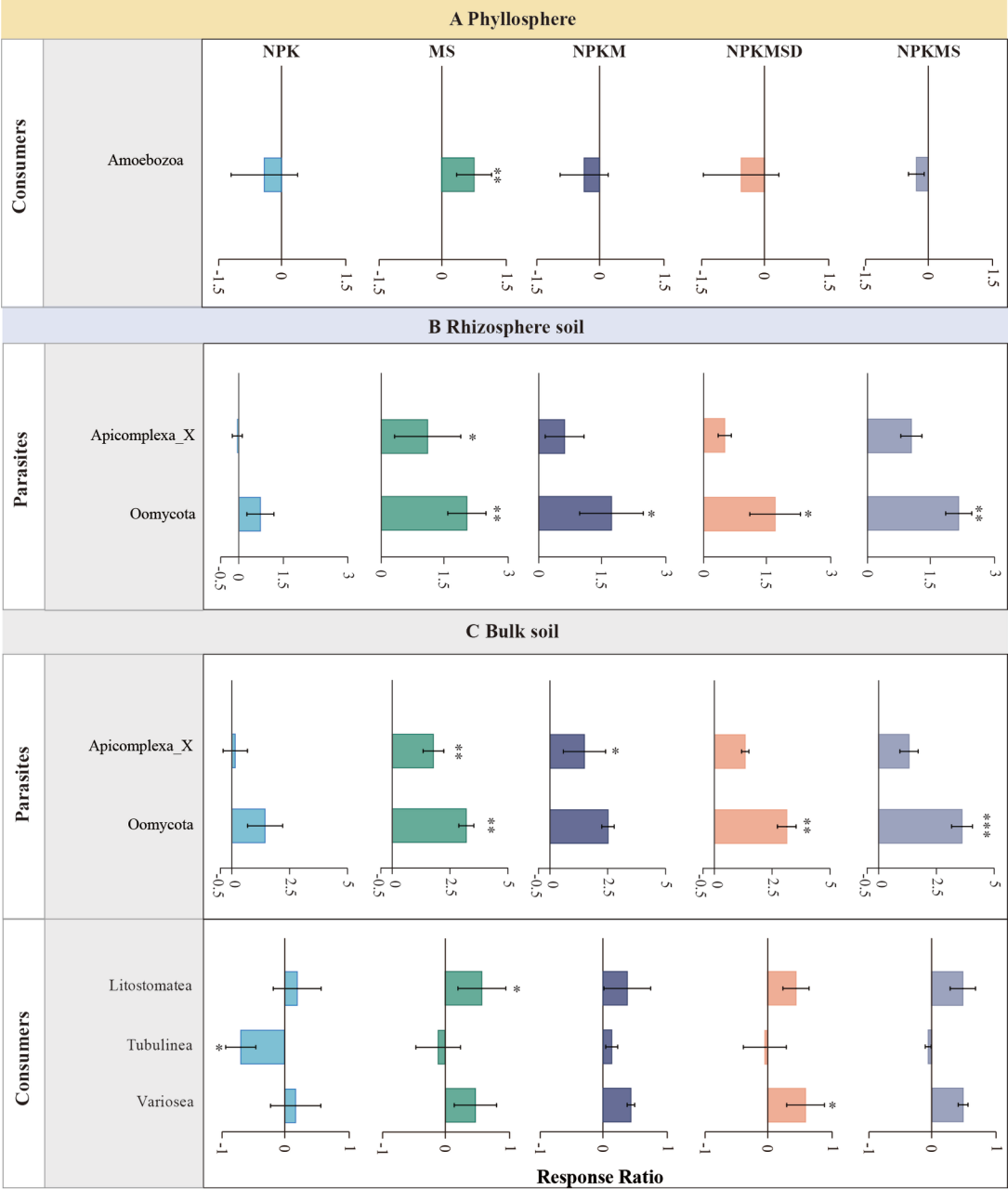
Figure 6 Structural equation modelling (SEM) showing the relationships between fertilization treatments, crop-associated compartments, soil properties and protistan, bacterial and fungal community compositions (A). Continuous and dashed arrows represent the significant and nonsignificant relationships, respectively. Adjacent number in the same direction as the arrow represents path coefficients, and the width of the arrow is proportional to the degree of path coefficients. Significance levels are denoted with $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. The total standardized effects of multiple factors on the bacterial, fungal and protistan community compositions (B).



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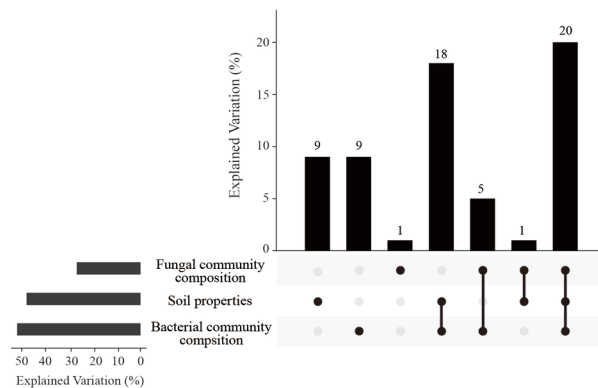


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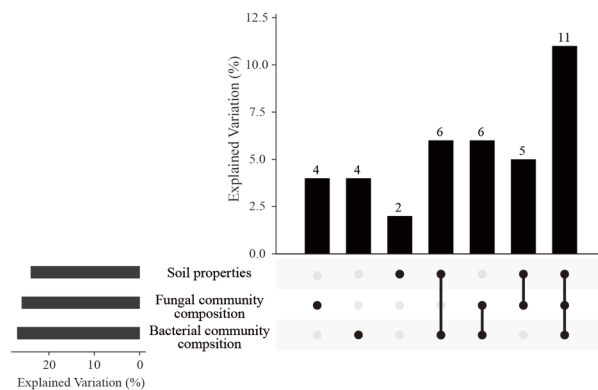


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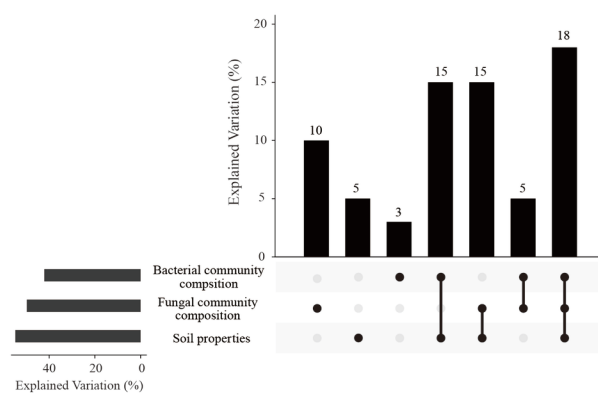
A Phyllosphere



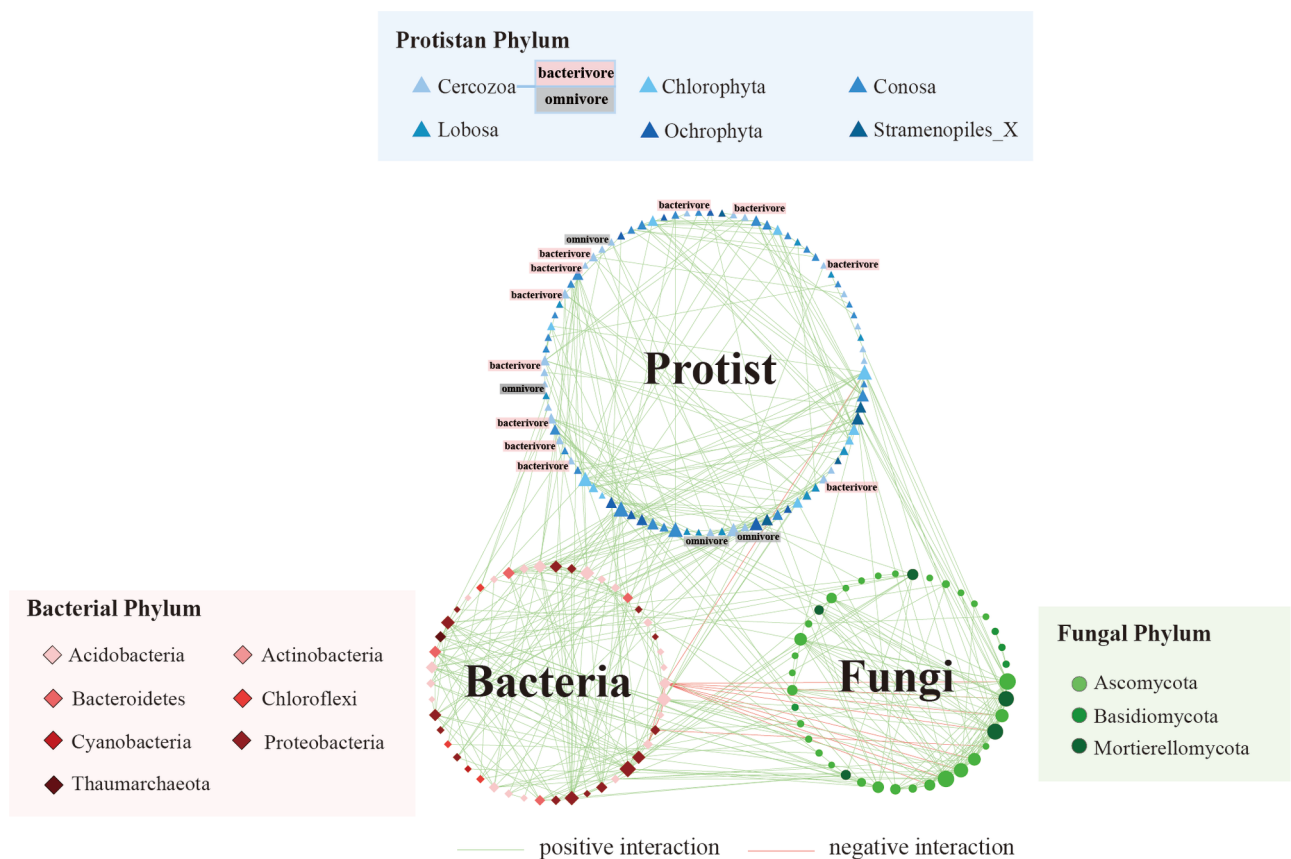
B Rhizosphere soil



C Bulk soil

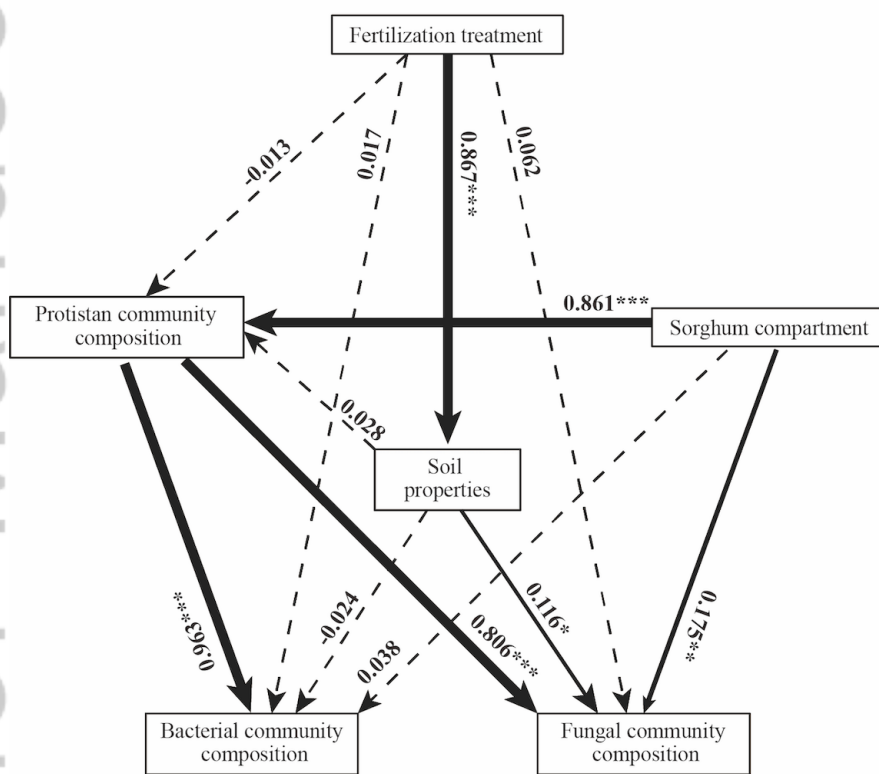


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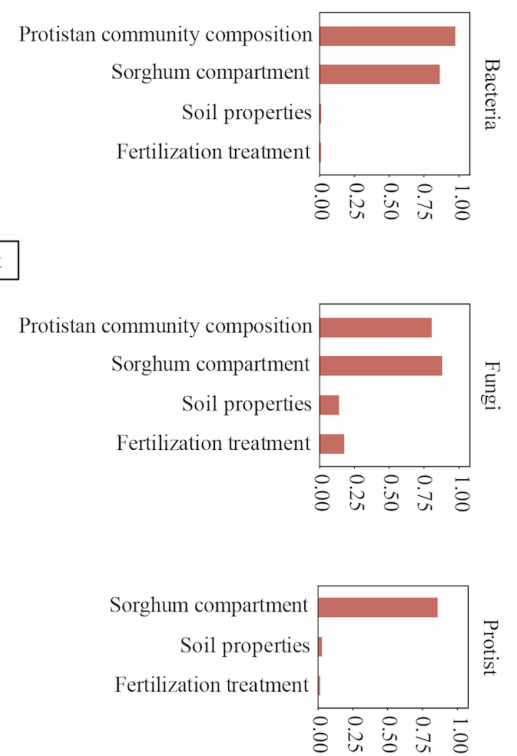
EMI_15385_Fig. 5.tif

A SEM model



$\chi^2/df = 0.02$, $P = 1.00$; GFI = 1.00, CFI = 1.00, RMSEA < 0.001

B Total effect



EMI_15385_Fig. 6.tif