Mixed Organic and Inorganic Amendments Enhance Soil Microbial Interactions and environmental stress resistance of Tibetan Barley on Plateau Farmland

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Abstract

Background

Sufficient crop yield while maintaining soil health and sustainable agricultural development is a global objective, serving a special challenge to certain climate-sensitive plateau areas. Despite conducting trails on a variety of soil amendments in plateau areas, systematic research is lacking regarding the influences of organic and inorganic amendments on soil quality, particularly soil microbiome. To our knowledge, this was the first study that compared the effects of inorganic, organic, and mixed amendments on typical plateau crop hulless barley (*Hordeum vulgare* L. var. *nudum*; also known as “Qingke” in Chinese) in Tibet over the course of tillering, jointing, and ripening.

Results

Microbial communities and their responses to amendments, soil properties, Tibetan hulless barley growth, yield, and their indicator microbial species were investigated. Results indicated that mixed organic and inorganic amendments promoted the abundance of rhizosphere microorganisms, enhancing the rhizosphere root-microbes interactions and resistance to pathogenic bacteria and environmental stresses. Additionally, values of total organic carbon (TOC) and carbon to nitrogen (C/N) ratio and the hullesss barley yield were the highest when mixed organic and inorganic amendments were applied. The rhizosphere microbiome demonstrated its unique adaptation to the plateau environment in carbon, nitrogen, sulfur, and phosphorus cycles, as well as resistance to environmental stresses. The 23 genes involved in plant growth and environmental adaptation in the mixed amendments treatments were significantly higher than other treatments.

Conclusions

Findings from this study suggest that the mixed organic/inorganic amendments can help establish a healthy microbiome and increase soil quality while achieving sufficient hulless barley yields in Tibet and presumably other similar geographic areas of high altitude.

Introduction

Tibetan hulless barley (*Hordeum vulgare* L. var. *nudum*), also known as “Qingke” in Chinese and “Ne” in Tibetan, is one of the earliest crops known to be cultivated by humans (3600 years ago) on the Tibetan Plateau [1, 2]. Tibetan hulless barley is also found in Europe, Central Asia, and East Asia. It has a robust tolerance to extreme environmental conditions such as drought, high salinity, cold, and high altitudes of 4000 m above sea level, including the Tibet Plateau [3–5]. Tibetan hulless barley is the main source of food and economic resources for local farmers, accounting for about 70% of grain crop in Tibet, as well as playing a vital role in the agricultural ecosystem [1, 6].

Inorganic fertilizer use continues to increase in the Tibetan Plateau to improve crop yields. Although it can supplement and replenish vital nutrients such as nitrogen, phosphorus, and other elements in soil, excessive application of inorganic fertilizers can cause loss of soil organic matter, increase greenhouse gas emissions from soil and soil acidification, and contribute to nitrogen contamination in groundwater [7–9]. Organic fertilizers/amendments start to gain attention as an alternative to traditional farming practices [10]. Farmland with organically-amended soils are known to have higher soil stability, better structure, and higher soil biological activities [11, 12]. The disadvantage of organic soil amendments are low nutrient input per weight and slow-release characteristics, as well as cost and application scale. Studies have been conducted on combining inorganic fertilizer and organic soil amendments to maintain soil health and achieve higher crop yields [13, 14].

Soil microorganisms are essential for soil and plant health and viability [15–17]. Studies indicate organic/inorganic amendments (fertilizers) significantly can increase the abundance, diversity, and composition of soil microbial communities [18, 19], where bacteria are significantly affected by amendment/fertilizer type, and fungi only respond to specific amounts of amendments/fertilizer. Organic amendments/fertilizers have been demonstrated to increase the relative abundance of functional taxa involved in heterotrophic and nitrogen fixation functions when compared to inorganic fertilizer treatments [20]. Mixed organic-inorganic amendments may change the structure of the soil microbial community and increase the activity of β-D-Glucosidase and proteas [19].

Different types of soil amendments also have an impact on interactions between microorganisms. For example, addition of organic fertilizer can reshape the microbial community and fortify resistance of wheat to soil-borne disease [21, 22]. And Xia et al. reported that the diversity of microbial communities in the rhizosphere decreased inversely with the level of inorganic nitrogen fertilizer [23].

Past studies were mostly conducted under greenhouse conditions or in the field in geographic areas with mild climates. Studies on the impact of organic and inorganic amendments on soil in Tibet, including its relationship to the rhizosphere and microorganisms at different growth stages, are lacking (Table S1). This study was conducted on the hulless barley farm in Tibet. Key parameters included: 1) impacts of organic and/or inorganic amendments on abundance, composition, function, and interactions of bacterial and fungal communities in the rhizosphere during three key growth stages (tillering, jointing, and ripening); and 2) how these interactions and impacts affect hulless barley growth and yield in Tibet.

Materials And Methods

Tillage experiment and sample collection

The field tillage experiment was conducted at the Institute of Agricultural Quality Standards and Testing, Tibet Academy of Agriculture and Animal Husbandry Sciences, Southern Agricultural Experiment Station of Nedong District, Shannan City, Tibet, China (28°57'N, 91°54'E, elevation 3967.3m) (Fig. S1a). The

Tibetan Plateau has a temperate monsoon climate, average annual temperature of 8.8 ℃, atmospheric pressure of 660.4 hPa, solar radiation of 6018.9 MJ, and a precipitation of 383.2 mm.

Tibetan hulless barley was planted from mid-April to early September 2020 with a growth period of about 135 days. The soil treatments consisted of three different amendment schemes: (1) DAP, inorganic fertilizer amendment; (2) ORG, organic soil amendment; and (3) MIX, a mixture of the inorganic fertilizer and organic soil amendments. The inorganic fertilizer was diammonium phosphate (Guizhou Kailin Group Public Co., Ltd., China), which was applied at a rate of 0.10 metric ton per hectare (mt/ha) for treatment (1). The organic soil amendment was a commercially available biochemically processed lignite called Ginate (Apaxfon Baotou BioScience and Technologies Co., Ltd., China), which was applied at a rate of 15 mt/ha for treatment (2). Ginate organic soil amendment is primarily composed of slow-releasing intermediate organic compounds, mineral-associated organic matter (MaOM), and to lesser extentreadily bioavailable short-chain organic compounds. This organic soil amendment was selected for its field performance in improving plant/crop growth as well ascontaining no pathogens, residual antibiotics, and weed seeds (which are known to be present in manures and manure composts). The MIX treatment (i.e., treatment 3) consist of the diammonium phosphate and ginate organic soil amendment, applied at rates of 0.05 and 7.5 mt/ha, respectively.

Each treatment was applied to separate fields (2m × 5m) with 1-m intervals between each field; Tibetan hulless barley was planted in each field. At each growth stage: tillering (mid-May), jointing (early July), and ripening (mid-August), six hulless barley plants were randomly sampled in each treatment (Fig. S1b). Sampling also included soil and rhizosphere (root zone) samples (the sampling time was based on the lack of precipitation 7 days prior to sampling). All samples were placed in an ice box immediately after collection and taken to the laboratory within 12h. The soil within 2 mm from the root surface was collected to serve as rhizosphere samples after gently shaking the roots to remove loosely attached soil clumps and brushing [24]. The bulk soil was used as soil samples after removing large particles and plant residues. A total of 216 samples including 108 rhizosphere soil and 108 bulk soil samples were obtained. Plant height was measured for each sampling stage and the crop was harvested in early September to measure thousand-grain weights and seed numbers per plant.

Soil chemical analysis

The soil samples were dried at 85°C for 48h and weighed to calculate the soil moisture (SM) content before being crushed and passed through a 2-mm sieve. Physical and chemical parameters of the soils, including pH, total phosphorus (TP), available phosphorus (AP), total potassium (TK), available potassium (AK), total nitrogen (TN), ammonia (NH_4^+), nitrate (NO_3^-), and nitrite (NO_2^-) were measured using standard soil testing procedures[25]. Soil base mineral ions (Si, Al, Fe, Ca, Na, Mg, As, Cr, Cu, Mn and Zn) were confirmed by using inductively coupled plasma optical emission spectroscopy (ICP-OES, SPECTRO BLUE, SPECTRO BLUE SOP, Germany). The soil total organic carbon (TOC) content was confirmed by using a high-frequency infrared carbon-sulfur analyzer (LECO CS744, LECO Corporation, Saint Joseph, MI, USA).

DNA extraction, qPCR, and shotgun metagenomics sequencing

DNA was extracted using E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) from 0.5g soil. The concentration and purity of DNA was found using 1% agarose gel and UV-Vis spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, DE, USA). Genomic DNA was stored in a -80°C freezer before subjected to high-throughput sequencing. Primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTC TAAT-3') and the primer pair ITS1F (5'-CTTGGTCATTTAGAGGAAGTAAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3') were used for qPCR, gene amplification and sequencing of region V3-V4 of the bacterial 16S rRNA gene and the ITS sequence, respectively. qPCR for 16S rRNA and ITS genes was performed on an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA) with a reaction mixture containing 10 µL of ChamQ SYBR Color qPCR Master Mix (2X) (Vazyme Biotech Co, Ltd., China), 1 µM of each primer, ~ 10 ng of total DNA, and ample RNase-free water to obtain a 20 µL volume. Standard curves were obtained with serial dilutions of a known amount of pMD18-T plasmid DNA containing a fragment of the target genes. Standard curves for 16S rRNA and ITS genes were based on serial dilutions of target genes (between 10^2 and 10^8 copies/ µL). These numbers were calculated from the concentration of extracted plasmids measured with a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Carlsbad, CA, USA).

The 16S rRNA and ITS genes amplification instrument are ABI GeneAmp® 9700 PCR thermocycler (ABI, Foster City, CA, USA) under the following conditions: initial transsexual at 95 ℃ for 3 min, followed by 35 cycles of denaturing at 95 ℃ for 30 s, annealing at 56 ℃ for 30 s and extension at 72 ℃ for 40 s, and single extension at 72 ℃ for 10 min. The PCR mixtures contained 5 × TransStart FastPfu buffer 4 µL, 2.5 mM dNTPs 2 µL, forward primer (5 µM) 0.8 µL, reverse primer (5 µM) 0.8 µL, TransStart FastPfu DNA Polymerase 0.4 µL, template DNA 10 ng, and ddH_2O to achieve a final volume of 20 µL. PCR reactions were performed in triplicates. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the standard instructions and quantified using a Quantus Fluorometer (Promega, Madison, WI, USA). The final amplicon obtained in the same molar amount of amalgamate and paired-end sequencing (2×250) was subsequently performed, using the Illumina MiSeq PE300 platform, in Shanghai Majorbio Bio-pharm Technology Co., Ltd., China.

DNA of each three out of six rhizosphere soil samples were pooled, and two pooled samples from each of the three treatments in ripening stage were used for metagenomic sequencing. For construction paired-end library, DNA was fragmented to an average size of about 300 bp using Covaris M220 according to standard instructions for metagenomic sequencing (Gene Company Limited, China). Paired-end library was prepared by using the TruSeq DNA Sample Prep Kit (Illumina, San Diego, CA, USA). Adapters containing the full complement of sequencing primer hybridization sites were ligated to the Blunt-end fragments. Paired-end sequencing was performed on the Illumina HiSeq3000 platform (Illumina Inc., San

Diego, CA, USA), using HiSeq 3000 PE Cluster Kit and HiSeq 3000 SBS Kits according to the manufacturer's instructions.

Sequencing data analysis

The amplicon sequencing data preprocessing was performed using Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1) software. The raw 16S rRNA gene and raw ITS rRNA gene sequencing reads were demultiplexed, quality-filtered by Fastp (version 0.20.0)[26], and merged by FLASH (version 1.2.11) [27] with the following criteria: (i) the 300 bp reads were truncated at each site, receiving an average quality score of < 20 over a 50 bp sliding window while truncated reads shorter than 50 bp or containing ambiguous characters were discarded (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of the overlap region is 0.2. Reads that could not be assembled were discarded; (iii) Samples were distinguished according to the barcode and primers and the sequence direction was adjusted for exact barcode matching and 2 nucleotide mismatches for primer matching.

The high-quality data was clustered into operational taxonomic units (OTUs) at a 97% similarity [28] with UPARSE (version 7.1, http://drive5.com/uparse/) and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by QIIME using a confidence threshold of 0.7 and the UNITE fungal ITS database with min-confidence of 0.75.

The metagenomic sequencing reads were first trimmed using Fastp (version 0.20.0) before being assembled into contigs using metaSPAdes [29] with default parameters. For each assemblies set, metagenome-assembled genomes (MAGs) were recovered by using MetaBAT2 [30], MaxBin2 [31] and CONCOCT [32]. All MAGs were pooled and dereplicated using VAMB [33]. As the final step of metagenomic binning, bin_refinement module of MetaWRAP pipeline was used to improve the quality of all the MAGs [34]. CheckM was used to estimate the completeness and contamination of MAGs. Total 404 MAGs were retained under the criterion > 70% completeness and < 10% contamination. Taxonomic assignment of MAGs was performed using GTDB-tk [35].

Gene prediction using PROKKA for these scaffolds and the predicted proteins were assigned to the KO using the KEGG Automatic Annotation Server [36]. Trimmed high-quality reads locating on the given scaffolds were counted to calculate the abundance of KOs in each sample using Burrows-Wheeler Aligner (BWA) [37]. The matrix was normalized by dividing the absolute amount of each functional gene by the total number of reads assigned to functional genes in each sample.

Statistical analyses and visualization

Statistical analysis of all data was performed using different software packages in R 3.6.2 for analysis and visualization [38]. Bray-Curtis distances were calculated using the "vegdist" of the R "vegan" package, A PCoA (principal coordinate analysis) ordination plot was then implemented using the "cmdscale" package. β diversity as well asthe similarity of sample subgroups and its impacts on the microbial community was tested using the "Adonis" package. The "randomForest" package was used to evaluate

soil properties and microbial metabolic processes sensitive to soil amendments; predictor variables were tested for significance with the "rfPermute" package. Wilcoxon rank-sum tests were performed using the "compare means" of the "ggpubr" package for soil properties, microbial biomass, and samples of Tibetan hulless barley yields for different soil amendment treatments. Mental correlation test of soil properties with bacterial and fungal communities was implemented using the "ggcor" package. In order to identify which microbes responded significantly to fertilizers, we performed indicator species analysis using the "multipatt" of the "indicspecies" package [39].

The "Hmisc" package was used to calculate the correlations between different grouped OTUs and Gephi (version 0.9.1, https://gephi.org/) was used to visualize the network patterns of different groups with significantly strong correlations ($\rho > 0.6$, $p < 0.05$). Topology data of networks was counted. The nodes with the top 1% connectivity were defined as keystone taxa.

In order to systematically understand the direct and indirect effects of fertilizers on microbial communities and plant production, partial least squares path modeling (PLS-PM) for different treatments were constructed using the "plspm" package. All factor loadings in the model are > 0.7, including soil properties, diversity of rhizosphere and bulk microbial communities, and traits of growth. PLS-PM path coefficients of three different fertilization groups are combined into a structural equation modeling structure (SEM). The "ggpmisc" package was used for regression analysis and visualization.

Results And Discussion

Influences of soil amendments on soil properties

The Tibetan hulless barley growth cycle was completed at approximately 135 days, during which physicochemical properties of soils from different amendment treatments varied significantly (Fig. 1 and Table S2). Random forest analysis identified that 5 soil physicochemical factors: pH, AP, TOC, C/N ratio, and Zn corresponded strongly to soil amendment treatment types (Fig. 1a). The TOC, C/N ratio, and pH were significantly higher in MIX and ORG groups than the DAP group; AP and Zn were significantly higher in the DAP group compared to the other two groups (Fig. 1b). The higher values of these physicochemical parameters most likely resulted from added soil amendments. For example, high organic matter content in the organic soil amendment resulted in higher amounts of TOC and C/N ratio while higher AP in the DAP group was due to the high P content of the corresponding inorganic fertilizer.

Data from the PCoA analysis (Fig. 1c) indicated that there were significant differences ($P < 0.001$) between the DAP, MIX, and ORG treated soil groups, three growth stages, and bulk and rhizosphere soils ($P < 0.01$). During the three growth stages, NO_2^- was significantly different in DAP, MIX, and ORG treated soils; TOC and levels of metal elements such as Fe, Al, and Zn changed significantly in DAP and MIX treated soil groups. In comparison, properties of the soil amended with organic fertilizer remained the most stable in terms of its parameters (Table S3). Additionally, values of the physicochemical parameters associated with nitrogen were significantly higher in organic amendments than that of bulk soil (Table

S4). This is attributable to the rhizosphere microorganisms that benefit plant growth by participating in the nitrogen cycle through processes such as nitrogen fixation [40, 41].

Influences of different soil amendments on farmland soil bacteria and fungi communities

Soil amendments are known to influence soil microbial communities, microbial biomass, composition, and diversity [42, 43]. During the hulless barley growth stage, the rhizosphere microbial biomass in the DAP treated soil decreased: bacterial biomass decreased from 4.19×10^9 copies·g^{-1} to 2.21×10^9 copies·g^{-1} and fungal biomass decreased from 5.06×10^8 copies·g^{-1} to 3.18×10^8 copies·g^{-1} (decreases of 47.2 and 37.2%, respectively). In contrast, the rhizosphere microbial biomass in the MIX treated soil increased: bacterial biomass increased from 1.91×10^9 copies·g^{-1} to 1.16×10^{10} copies·g^{-1} and fungal biomass increased from 3.64×10^8 copies·g^{-1} to 6.02×10^9 copies·g^{-1} (increases of 507 and 65.4%, respectively) (Fig. 2a). The bacterial and fungal biomass in the ORG treated soil increased by 202% and 90.5%, respectively.

The microbial biomass of the DAP treated soil peaked during thee tillering stage and decreased following hulless barley growth. In contrast, the microbial biomass in ORG and MIX treated soils increased with crop growth. Inorganic fertilizers tend to be less stable in soils over time and are readily consumed, mobilized, and leached out. Organic soil amendments exhibit slow-release characteristics that last over a longer period of time [10, 12]. This long-term supply of organic carbon and nutrients optimally benefit and sustain microbial biomass growth and activities.

Densities of the rhizosphere bacterial and fungal biomasses were significantly higher in the treated soil compared to bulk soil. During the ripening stage, the difference between the bulk and rhizosphere bacteria biomass in MIX treated soil was approximately 9×10^9 copies·g^{-1}; other changes in microbial biomass did not exceed 3×10^9 copies·g^{-1}. Changes in fugal biomass were minimal (Fig. 2a). Through 16S rRNA and ITS gene sequencing of 108 soil samples, 6877 bacterial OTUs and 2597 fungal OTUs were analyzed. The data indicates that mixed organic and inorganic soil amendments increase the diversity of microbial communities in the rhizosphere (Fig. S2). The diversity of microbial communities, particularly for bacteria in bulk soil has a higher level of response to soil amendments compared to rhizosphere microbial communities. Lower diversity was observed for fungi. This response was similarly observed in other studies connecting microbial community responses to various soil amendments [44, 45]. The dominant bacteria phyla are Actinobacteriota, Bacteroidota, Firmicutes, and Proteobacteria; dominant fungi phyla are Ascomycota, Basidiomycota, and Mortierellomycota. At the genus level, the relative abundance of top 20 dominant bacteria are *Arthrobacter, Sphingomonas, Chitinophagaceae, Thermomonas, Paeniglutamicibacter*, and *Rhodanobacter*,dominant fungi genera are *Gibberella, Didymella, Plectosphaerella, Naganishia, Cystofilobasidium*, and *Cladosporium* (Fig. S3). The composition of bacterial and fungal communities exhibit significant differences across amendment/fertilizer treatments (Fig. 2b). The highest differences are seen between different soil

amendment treatments and sampling locations in bulk and rhizosphere soils, most sensitively to early Tibetan hulless barley growth.

PCoA further identified the effect of different soil amendments on bacterial and fungal communities (Fig. 2c). Both bacteria and fungi were separated into different groups based on treatment, time, and site characteristics. PCoA indicate that fungi communities were significantly impacted by soil amendments and to a lesser degree rhizosphere bacterial communities across the stages. Based on Bary-Curtis distance, fungi communities are more sensitive than bacteria communities to rhizosphere-bulk soil samples and soil amendment treatments (Fig. 2d). Relative to DAP treated soils, rhizosphere bacteria communities are similar between ORG and MIX treated soils. The similarity stems from early stage of crop growth, where microorganisms originating from the bulk soil form a new rhizosphere microbiome [46–49]. The correlation between bacterial and fungal communities and environmental factors was analyzed (Fig. 2e), the composition of the bacteria community was correlated with SON and TP and the fungi community with TOC and trace metal elements (Mg, Cu, Al, and Mn). Both bacteria and fungi communities were possibly influenced by TOC, SON, TP, Zn, and Mg in soil. These environmental factors are consistent with the analysis of soil physicochemical factors (Fig. 1a). Nitrogen and phosphorus were possible limiting factors for bacterial growth in Tibetan hulless barley farmland soil. Fungi were possibly impacted by the presence of TOC and trace metal elements in fertilizer. It was reported that bacterial metabolism altered nitrogen-phosphorus synergy while percentage of fungi increase with higher of organic carbon content [50, 51].

Amendment microbial indicators in Tibetan Hulless Barley farmland soil

A total of 210 bacterial OTUs (1.76% of total soil community sequences) and 121 fungal OTUs (3.95% of total soil community sequences) were significantly altered ($p < 0.05$) by soil amendment treatments across growth stages. The primary bacteria phyla were Actinobacteriota, Chloroflexi, and Firmicutes. The primary fungi phyla were Ascomycota, Basidiomycota, and Mortierellomycota. For bulk soil, a total of 247 bacterial OTUs (3.54% of total soil community sequences) and 131 fungal OTUs (3.51% of total soil community sequences) were identified ($p < 0.05$) as indicators across fertilizer treatments. Several functional microbiotas responded strongly to soil amendment treatments (Fig. 3). In contrast, soil amendments have a diminished effect on abundant microbiota species due to their strong resistance functionalities [42, 52, 53]. The number of indicator species in rhizospheric soil was marginally lower than that of bulk soil due to plant growth and soil amendment impacts on the microbiome and rhizosphere. For example, rhizosphere bacteria genera *Angustibacter* degrade gelatin and aesculin, *Flavobacterium* degrades Casein and gelatin for auxin production and P-solubilization[54], and *Gemmatimonas* modulates C and N intakes depending on environmental stimuli[55]. Fungal genera identified as indicator species are represented by *Saprotroph* and other guilds. The effects of soil amendments on the rhizosphere and bulk soil bacteria and fungi vary. Rhizospheric soil amendment indicator species possess metabolic functions promoting plant growth while bulk soil indicators lack these functions.

A total of 44 bacterial OTUs (13.47% of total soil community sequences) and 41 fungal OTUs (13.87% of total soil community sequences) were identified as growth stages indicator species (relative abundance > 0.1%, $p < 0.05$). These values were lower than the number of indicator species based on soil amendment treatments (Fig. S4). A total of 25 bacterial OTUs (9.65% of total soil community sequences) and 14 fungal OTUs (5.03% of total soil community sequences) were identified in bulk soil. These values were lower than the abundance of indicator species present during rhizospheric growth as well (relative abundance > 0.1%, $p < 0.05$). Plant growth-promoting rhizobacteria (PGPR) is the indicator species that changes significantly across Tibetan hulless barley growth stages, allowing for better plant adaptation to environmental stressors; for example, the relative abundances of *Arthrobacter*, *Devosia*, *Bacillus*, and *Oceanobacillus* (Fig. S4). Previous studies on wheat or barley (Hordeum vulgare L.) include these genera as significant PGPR indicators[56–58]. These plant growth indicator species have high relative abundances (reddish in heatmap color, Fig. S4) and their metabolic functions play an important role in plant growth and soil nutrient cycling. The number of plant growth indicators was low, but the abundance was high.

Soil amendment effects on microbial co-occurrence patterns

Co-occurrence network analysis examines interactions between species. The microbial community of the MIX treated soil has the most complex microbial co-occurrence pattern with the highest node and edge counts as well as clustering coefficient; it has the lowest ratio of negative to positive connections (Figs. 4a-b and S5). This indicates that microbial cooperation is dominant, improving the metabolic efficiency of microorganisms and the utilization rate of nutrients by plant uptake as a result [59]. The percentages of bacterial and bacterial connections compared to total connections was > 80%, connections between bacteria and fungi > 10%, and between fungi and fungi between 1% -2%. The MIX treated soil had most bacterial-fungal connections, accounting for 16.4% of the total number of connections (Figs. 4b-d). Most nodes species are present in MIX treated soil bacteria phylum Actinobacteriota and fungus phylum Ascomycota are dominant (Figs. 4c-e). Microorganisms with top 20 connectivity were considered as key species. The MIX treated soil has the highest average number of important species connections, followed in descending order by the ORG treated soil with the DAP treated soil possessing the least connections (Figs. 4c and 4d). Key genera *Mizugakiibacter*, *Granulicella, Planococcus, Mizugakiibacter*, and *Planococcus* have inhibitory and resistance effects on plant diseases while *Granulicella* promotes plant growth as well [60, 61].

The co-occurrence patterns of different fertilizer treatments corresponding to the growth stages of Tibetan hulless barley show that the tight complexity of interactions gradually resulted in a decrease across growth stages (Fig. S6). The complexity of the network structure for fungal indicators increased from tillering to jointing growth stage before decreasing in the ripening growth stage. The connectivity of each network varied across the growth stages as well. Bacterial interaction structure varied while fungal interaction structure exhibited a slow-release response time. This was due to the bacteria having a shorter

turnover time and responding more rapidly to environmental changes while fungi show resistance to environmental changes, resulting in a longer period of growth turnover time [19, 62].

The combined application of organic and inorganic soil amendments significantly increases the abundance of key species, major soil ecological groups in relation to key taxon, and improves interrelated interactions between microbes and their functionalities. These complex and tight interactions can enhance the utilization of resources and resistance to environmental stressors and disease, thus promoting the healthy growth of Tibetan hulless barley in an inarable plateau environment [17, 63–65].

Correlation between microbial communities and soil amendment treatments

Plant traits showed that there was no significant difference in plant heights across soil amendment treatments during the tillering stage; plant heights fromDAP soil amendments were significantly lower compared to the other soil amendment treatments during the jointing and ripening stages (Fig. 5). Thousand Grain Weight (TGW) among the three soil amendment treatments were ranked MIX > ORG > DAP; a similar trend was observed for number of plants (Fig. 5 and S7a). The estimated yield was highest in the ORG treatment with no significant difference between DAP and MIX treatments. The MIX treatment achieved the most optimal growth and crop yield (546, 973 kg/km^2) (Fig. 5a and S7a).A total of 9 OTUs from rhizosphere microorganisms share a significant correlation with TGW and plant height (Fig. 5b; $\rho > 0.5$, $p < 0.05$). These are as follows: bacteria OTU216 (*Devosia*, 0.71%), OTU913 (*Nocardioides*, 0.04%), OTU3105 (*Cereibacter*, 0.03%), OTU4330 and OTU4827 (*Brachybacterium*, 0.06% and 0.03%), OTU4942 (*Flavisolibacter*, < 0.01%), OTU4686 (norank family Caloramatoraceae, < 0.01%), OTU6855 (norank family Vicinamibacteraceae, 0.01%), and fungi OTU2172 (*Mortierella*, < 0.01%) (Fig. 5b). The number of microorganisms involved in a positive correlation with plant height did not vary significantly across soil amendment treatments (3 from DAP, 2 from MIX, and 1 from DAP & ORG). Species correlation with Tibetan hulless barley yield, grain number, and plant number show that most plant growth indicator species share a significant positive correlation with plant height and enriched in the jointing stage (Fig. S7b). The soil amendment indicator and abundant species share a notable positive correlation with grain counts. These species mostly belonged to the MIX group and ORG group, with a small portion belonging to the DAP group (Fig. S7c). Compared with different growth stages, highly sensitive bacteria belonged to phylum Proteobacteria. Bacteria responsive to soil amendment treatments mostly belonged to phyla Actinobacteria and Ascomycota (Fig. 3).

The PLS-SEM model showed that the effect of soil amendment treatments on soil pH was most observable among environmental factors. The analysis interpretation (R^2) of soil organic matter sources show that MIX treatment had the most significant positive effect on the increase of soil organic matter. The growth of bacteria and fungi was positively affected by soil organic matter. Soil properties (pH, inorganic matter, organic matter) had a stronger effect on the rhizosphere bacterial community than fungi community. It indicates that bacteria are more sensitive to fertilization than fungi during plant growth.

The MIX treatment contributed the most to changes in soil organic concentration for the microbiome as well as the growth and yield of Tibetan hulless barley (Fig. 5c).

The health and quality of farmland is driven by soil physicochemical factors and the microbiome in relation to rhizosphere microorganisms. Through linking soil amendment treatments with soil physicochemical parameters, growth stages, and the microbiome, we can establish a relationship between the response model of the complex soil-microbe-crop system and Tibetan hulless barley. Specific microbial groups from the soil around the root zone of Tibetan hulless barley, more specifically unique bacteria including *Arthrobacter*, *Microbacterium*, *Planomicrobium*, *Nocardioides*, and so on were optimally enriched. Compared to fungi, bacteria are more diverse in their response. However, fungi can more robustly maintain ecological plateau farmland systems. Rhizosphere microorganisms form complex interactions and are subjected to a dynamic process impacted by host type, plant growth environment, and physiological status [41].

The relationship between Tibetan Hulless Barley rhizosphere microbiome and metabolic functions

The metagenomic data was effectively assembled in 18 relative high quality draft MAGs (Table S5). MAG coverage of the dominant and representative rhizosphere bacteria (*Nocardioides*, *Rhodanobacter*, *Sphingomonas*, *Mycobacterium*, *Flexivirga*, and so on) helps us to infer presence and absence of pathways and understand comprehensive metabolic functions of rhizosphere microorganisms. In carbon biodegradation pathway, chitin-degrading genes degrade inactive carbon in the soil, replenishing the supply of carbon sources under extreme environments and conditions [66]. Rhizosphere microbial functions during carbon fixation, such as glycerol utilization, pyruvate metabolism, formate metabolism, ethanol fermentation, and acetyl-CoA metabolism are all related to the rTCA cycle. This cycle is prevalent in anaerobic bacteria that thrive in the low-oxygen conditions of the Tibetan Plateau. A large variety of acetic acid fermentation genes were discovered as well. There is increasing evidence that acetic acid production plays an important role in the organic carbon cycle in extreme microaerobic or anaerobic habitats [67]. Nitrogen cycling driven by the rhizosphere microbiome is important for crop growth [45]. Assimilation and dissimilatory nitrate reduction and glutamate synthesis are both related to the conversion of ammonia into organic nitrogen and an ammonia transporter helps plant roots absorb ammonium nitrogen [68, 69]. In extreme environments, Tibetan hulless barley rhizosphere microorganisms can synthesize large amounts of amino acids for resistance against environmental stressors. As a result, a consistent input of ammonia is crucial for a thriving microbial community. Assimilatory sulfate reduction consumes sulfate in the environment to synthesize sulfur-containing amino acids. Cysteine can be degraded and catalyzed by cysteine desulfhydrase to generate hydrogen sulfide, pyruvate, and ammonia-all of which possess regulatory effects on plant growth [70, 71]. Phosphorus metabolism includes inorganic phosphorus, polyphosphoric hydrochloride, phosphate, and organic phosphorus metabolism. Energy is released, promoting plant resistance to harsh environmental stressors and inhibitors to growth [72]. Additionally, some functional genes in rhizospheric

microorganisms such as heat shock protein genes, genes related to heavy metal resistance, and genes that resist oxidative stress help plants adapt to harsh environments and promote plant growth as well.

Fertilizers promote changes in microbial abundance and significantly impact various metabolic functions such as carbon, nitrogen, phosphorus, and sulfur cycles (Fig S8). Genes in higher abundance within the MIX treatment include fructose and mannose metabolism genes *mtlK*, *pfp*, and *mtlA* in the carbon degradation pathway; TCA cycle genes such as pyruvate metabolism genes *fumA* and *fumB;* glyoxydicarboxylic acid metabolism genes *acnB* and *PCCA* in the carbon fixation pathway; and acetate fermentation gene *aldH*. This indicates that the microbial community of the MIX treatment group has more carbon source pathways. Additionally, nitrate reduction genes associated with the nitrogen cycle, *nrtC* and *narB* as well as amino acid metabolism-related genes (arginine metabolism genes *GDH2* and *ureC*, cysteine and methionine metabolism genes *gshB*, and valine, leucine, and isoleucine metabolism genes *vdh* and *accD6)* were more abundant in the MIX treatment group. Ammonia was likely converted into organic nitrogen, which is beneficial for crop production [73].

Sulfur-related sulfate-reducing genes *cysA* and *ssuD* are more abundant in the MIX treatment group and could fix arsenic, reducing its effectiveness in rice plants and providing an effective way to inhibit the infiltration of arsenic into the food chain [74]. The abundance of nucleotide metabolism-related gene *xdhB*, cofactor and vitamin metabolism-related genes *cobN* and *cobIJ*, terpenoid metabolism-related gene *atuF*, and lipid metabolism-related primary bile acid genes *AMACR* and *mcr* in the MIX group is higher compared to the other treatment groups. Vitamins and terpenoid metabolites play an important role in promoting the production of essential compounds in plants and bacteria, inducing resistance to pathogens and promoting plant growth as a result[75, 76]. Additionally, primary bile acids contribute to crop disease resistance [77]. Therefore, mixed fertilizer can be used optimally to promote Tibetan hulless barley resistance to negative plateau environmental stressors and inhibitors to plant health.

Conclusions

Application of soil amendments to soil can effectively increase crop yields and optimize soil quality, primarily in regards to organic matter content. In extreme ecological environments like the Tibet Plateau, quality farmland is scarce and key soil viability parameters such as organic matter and microbial populations are more vulnerable and sensitive to climatic changes. In Tibetan soil, microbial communities, specifically rhizospheric microorganisms, play an even more important role in crop growth, nutrient absorption, disease resistance, and sustaining a healthy and regenerative garniture. Data from this study suggests that the treatment of mixed inorganic and organic soil amendments can achieve the greatest hulless barley yield and establish healthy soil. Inorganic fertilizer only (DAP) treatments result in a short-term and rapid rise of soil nutrients level (N and P) as well as the population density of rhizospheric microbes; organic amendment only (ORG) treatments promote a higher abundance of microorganisms in the rhizosphere that favors beneficial, growth-promoting bacteria that are essential for the growth of Tibetan hulless barley. Bacteria and fungi show phase differences in their rhizosphere in response to fertilizer treatments. This indicates that soil amendment effects on farmland systems should

be studied over a long-term scale (multiple years). The key to establishing an optimal microbiome in the rhizosphere is the balance between bacterial sensitivity and fungal stability. Mixed ORG and DAP amendments appear to have achieved those goals during the study period. However, longer-term performance can provide further confirmation through examining the intricacies of more growth cycles and seasons of farmland systems.

Declarations

Availability of data and materials

Raw amplicon sequence data related to this study were deposited in the NCBI Sequence Read Archive (NCBI SRA) under bioproject PRJNA835221.

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Authors' contributions

XG, SL and JF were responsible for conception and design of the research; YL, LH and CQ collected samples; YL performed DNA extractions, measured and analyzed the environmental factors; YL, ZC, RZ, ZG and TZ performed sequence assembly, annotation, analysis and visualized Figures; WS and LC did some statistical analysis and submitted sequence data; XG, ZC and YL contributed to the writing of the manuscript; SJ and JW revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Ethics declarations

1. Ethics approval and consent to participate

Not applicable.

2. Consent for publication:

Not applicable.

3. Competing interests

The authors declare that they have no competing interests.

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Figures

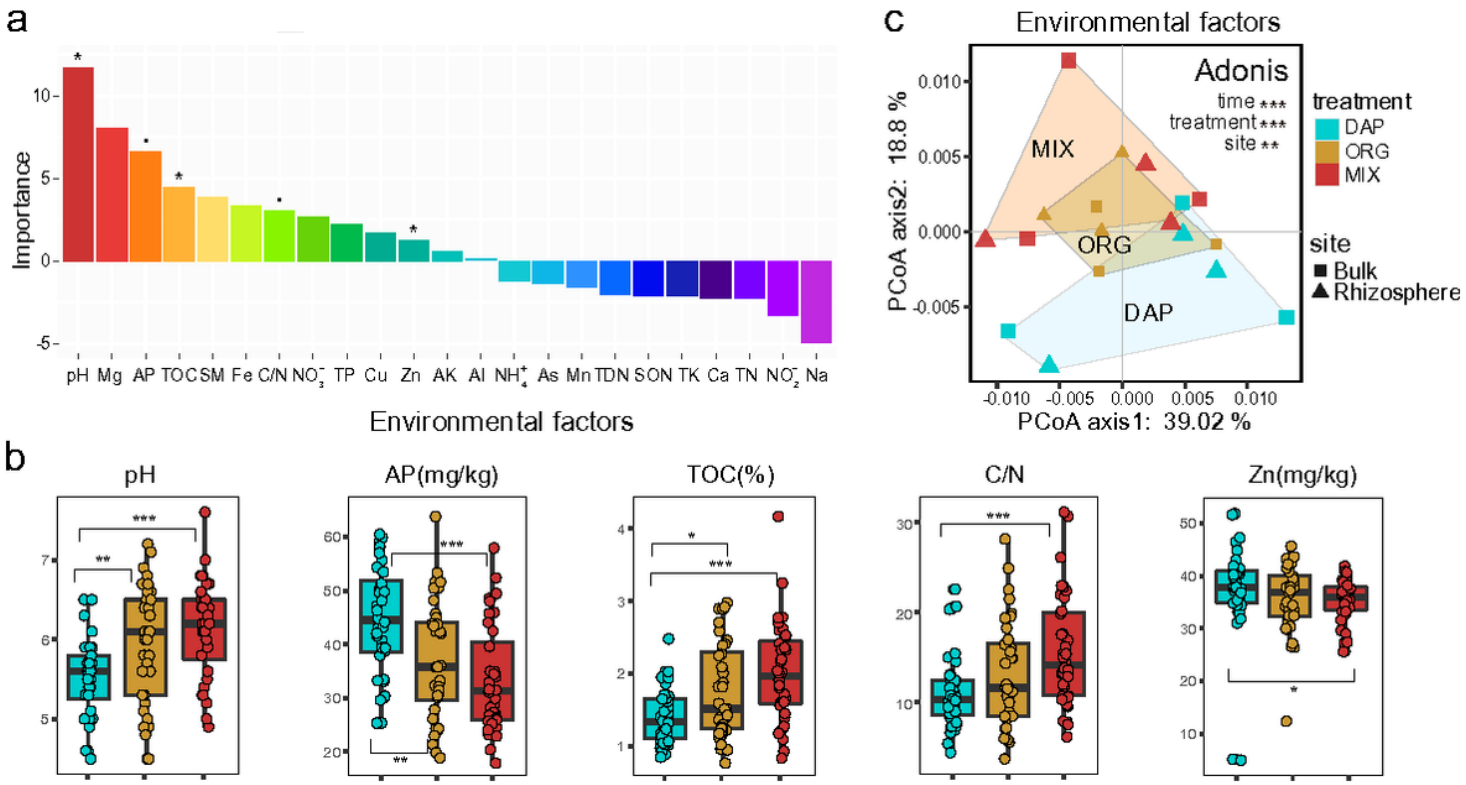

Figure 1

Soil physicochemical parameters in soils from the Tibetan hulless barley farmland where the inorganic (DAP), organic (ORG), and mixed inorganic-organic (MIX) soil amendment treatments were applied.

a. Soil physicochemical predictors responses to soil amendment treatments determined by random forest analysis (*, p<0.1; *, p<0.05). **b.** Soil physicochemical factors (parameters) and responses to different soil amendments (Wilcoxon rank-sum test *, p<0.05, **, p<0.01, ***, p<0.001). **c.** Principal coordinate analysis of soil physicochemical factors (parameters) for all soil samples.

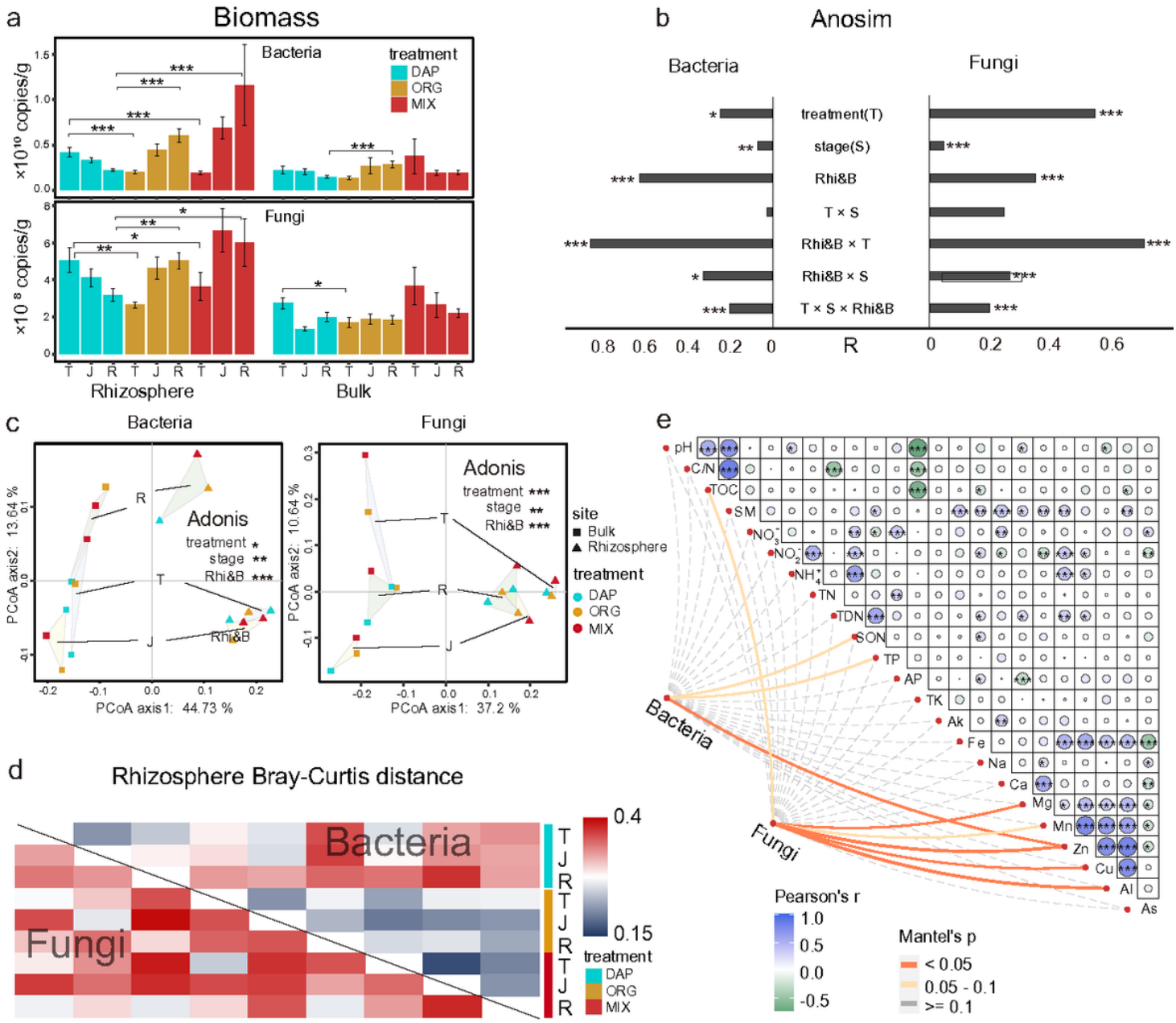

Figure 2

Microbial communities of inorganic (DAP), organic (ORG), and inorganic and organic mixed (MIX) soil amendments in Tibetan hulless barley farmland soil.

a. The bacteria and fungi biomass. **b.** Anosim analysis for the microbial community in rhizosphere (Rhi) and bulk (B) soil samples for the three Tibetan hulless barley growing stages for three r treatments**. c.** Principal coordinate analysis (PCoA) with Adonis (Permutational multivariate analysis of variance, PERMANOVA) tests for rhizosphere (Rhi) and bulk (B) soil samples in tillering (T), jointing (J) and ripening (R) growth stages of three treatments. **d.** Intergroup variance distance of bacterial and fungal communities in T, J, and R stages of three treatments for rhizosphere soil based on Bray-Curtis distance.

e. Correlation analysis of soil physicochemical factors with mantel tests conducted between bacterial and fungal communities (*, p<0.05, **, p<0.01, ***, p<0.001).

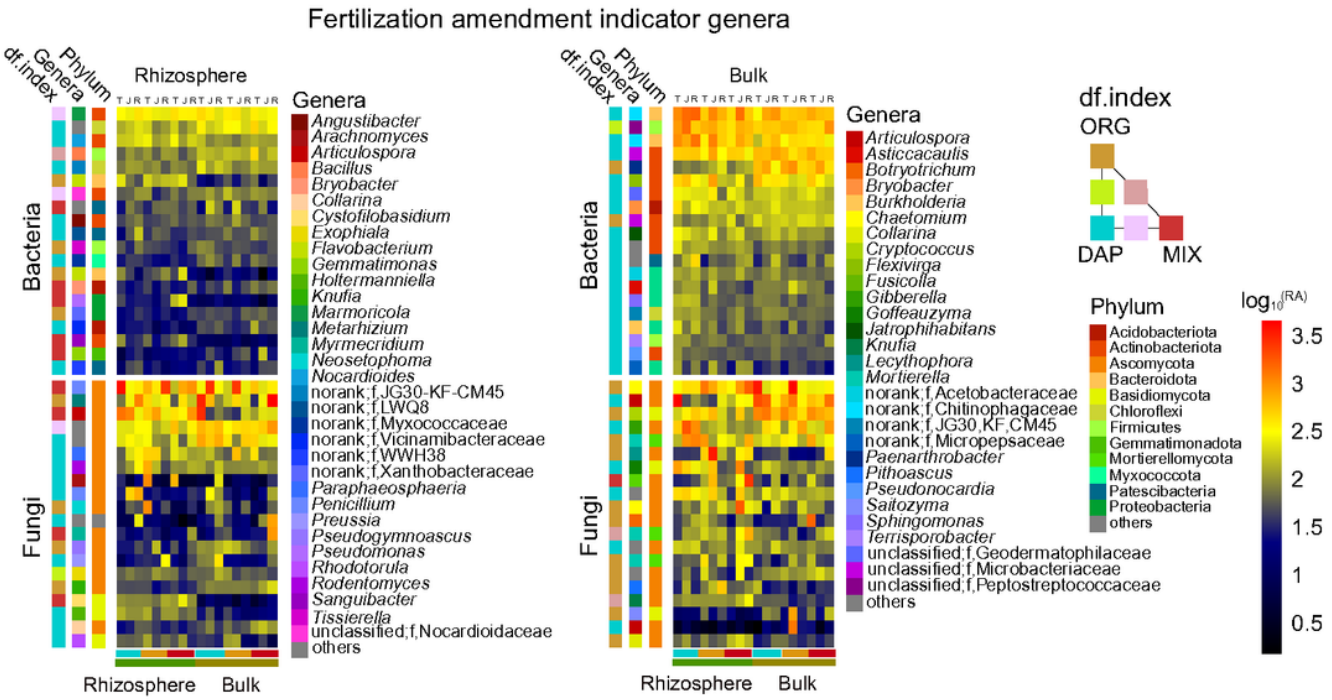

Figure 3

Abundance of rhizosphere (left) and bulk (right) microbial indicators in Tibetan hulless barley farmland soil.

The color bars on the left side of each heat map represent the differential enrichment of phylum and genera (represented by the legend in the bottom right corner of df. index, the different color blocks represent the enrichment of the OTU group in different fertilizer treatments. The color on the connection line between treatment group blocks indicates enrichment in more than one treatment); the upper and lower parts of the heatmap represent the relative abundance of bacteria and fungi, respectively.

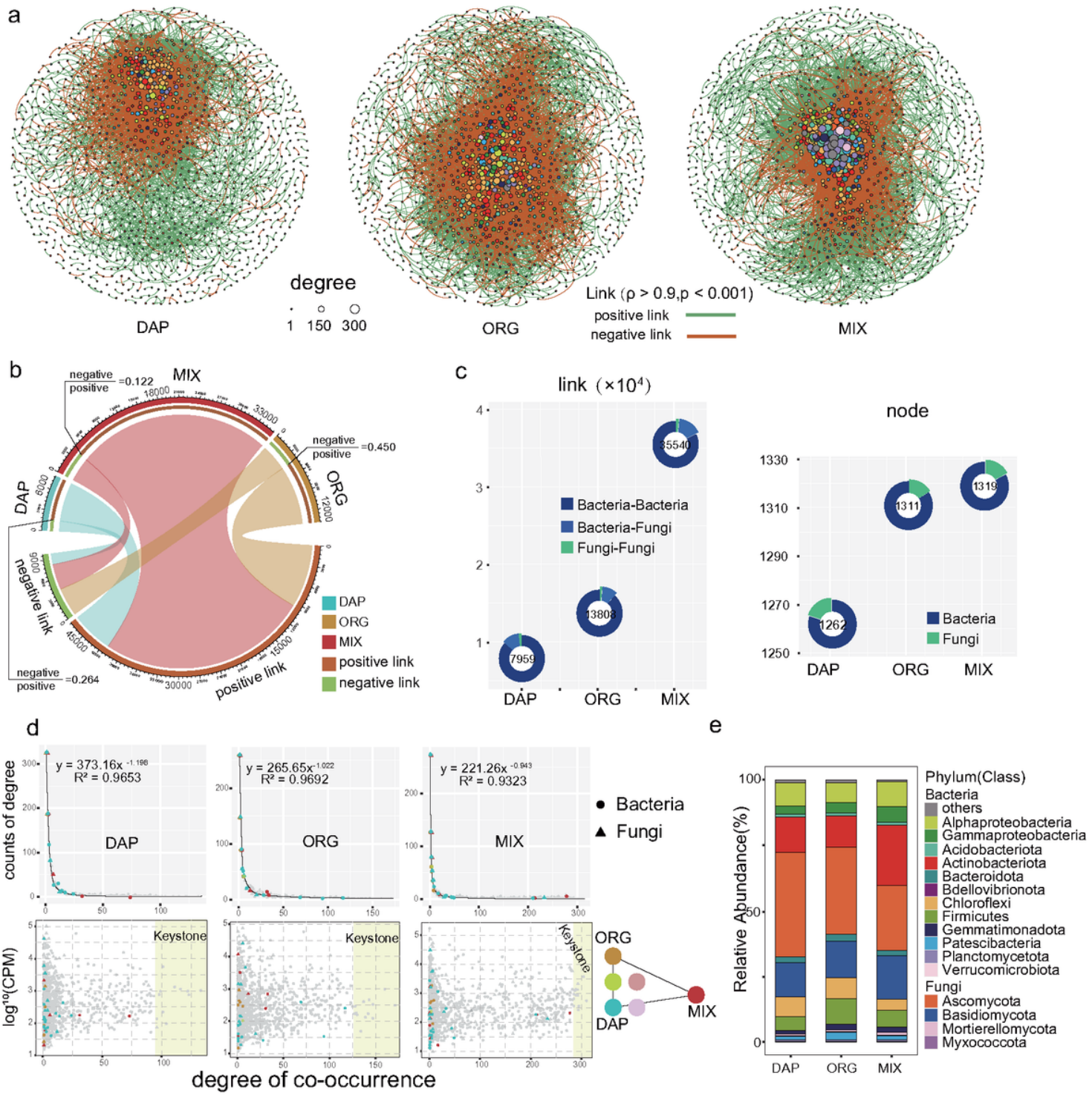

Figure 4

Interaction patterns of rhizosphere microbial communities from Tibetan hulless barley farmland soil with different soil amendment treatments.

a. Co-occurrence network of abundant OTUs (>0.1%) and soil amendment indicator OTUs (<0.1%) in the rhizosphere microbial community of inorganic (DAP), organic (ORG), and inorganic and organic mixed (MIX) soil amendment treatments (ρ > 0.9, p < 0.001; orange lines mean positive correlations between

bacteria, green lines mean negative correlations between bacteria), the color of node OTUs represents different phyla, and the size represents connectivity. **b.** The proportion of positive and negative connections of bacteria in three soil amendment treatments. **c.** Connections between fungi and bacteria across three soil amendment treatments. **d.** keystone bacteria and fungi across three soil amendment treatments based on the connectivity (top 20) in co-occurrence networks. **e.** Taxon classification of nodes at the phylum level within networks across the three soil amendment treatments.

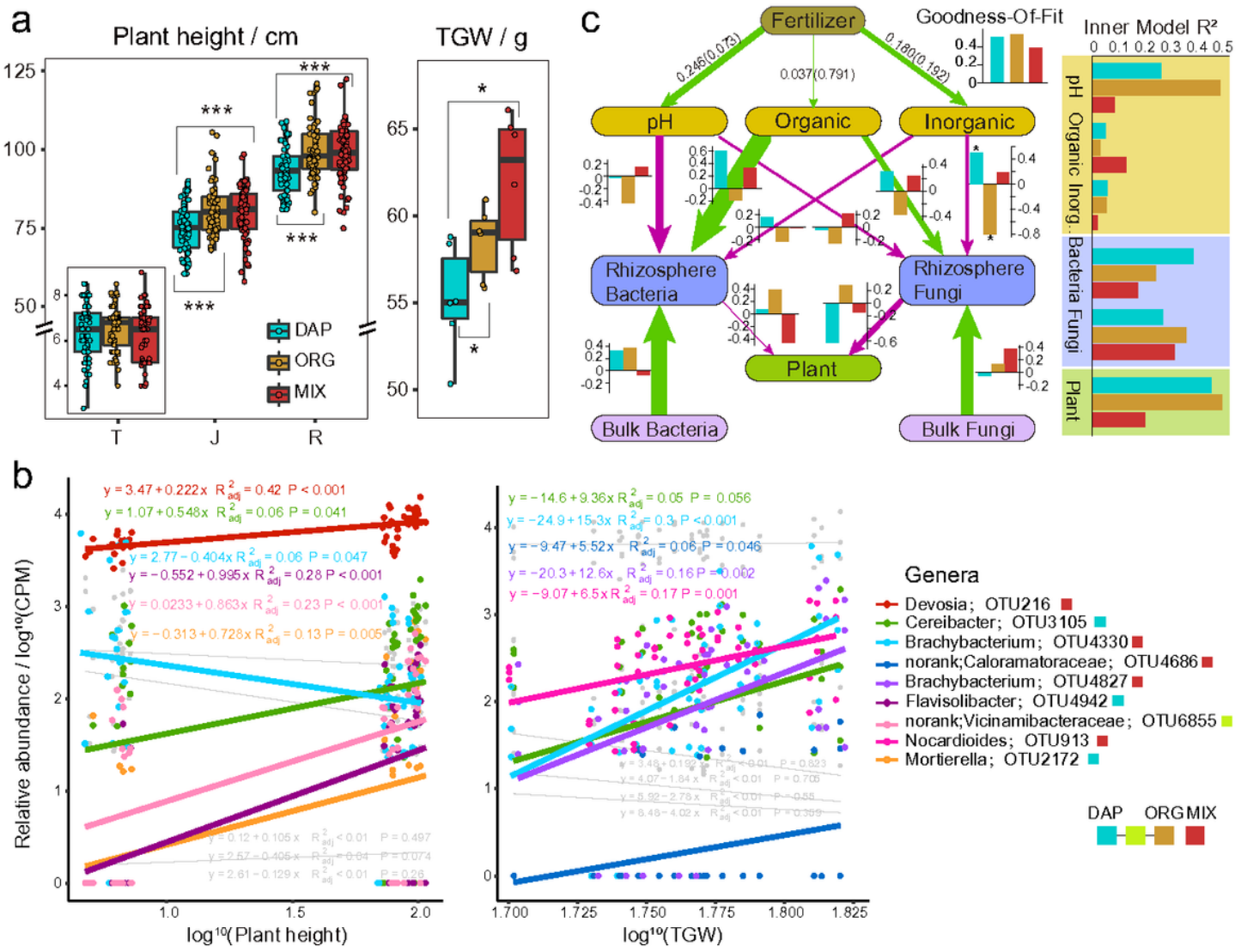

Figure 5

The growth traits of Tibetan hulless barley and interaction relation factors of microbial communities and physicochemical factors of farmland soil with applied inorganic (DAP), organic (ORG), and inorganic and organic mixed (MIX) soil amendment treatments on the Tibetan Plateau.

a. Plant height and thousand-grain weight (TGW). **b.** Correlated species with plant height and TGW, significant correlation between the relative abundance of indicator (Fig. 3) and keystone species in the network across soil amendment treatments (Fig. 4), the value is not significant if displayed in gray. **c.**

Partial least squares structural equation modeling (PLS-SEM) examined the impacts of soil amendment treatments on soil properties (pH, C/N, TOC), soil inorganic components (TN, NH_4^+, TP, AP, AK nutrients), rhizosphere and bulk soil microbial communities (bacterial and fungal richness and Shannon index), and direct or indirect effects of hulless barley traits (the column chart on the right shows the coefficients of determination "R^2" of the inner model). The width of the arrow describes the size of the average path coefficient (three types of fertilizer treatments), the green and red arrows represent positive and negative effects, respectively, and the bar graph represents the path coefficient of soil amendment treatment groups (*, P <0.05). the GOF (Goodness Of Fit) represents the model prediction effect.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 0426Supplementaryinformation.docx
- 0426TableS5.xlsx