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Combined Effect of *Bacillus subtilis* and Arbuscular Mycorrhizal Fungus *Rhizophagus irregularis* on Rhizosphere Microflora of *Achnatherum splendens* Under Drought Stress

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Abstract

The efficacy of microbial inoculants in enhancing plant drought tolerance is context-dependent, varying with drought severity and the dynamic interactions within the plant–soil microbe system. We conducted a two-factor pot experiment using *Achnatherum splendens* under three moisture regimes (well-watered (control), moderate drought, and severe drought) and four inoculation treatments (sterile inoculum (control), *Bacillus subtilis* (BS), arbuscular mycorrhizal fungi (AMF), and their co-inoculation (MIX)). The resulting 12 treatment combinations were each replicated four times. Drought stress significantly reduced both shoot and root biomass, disrupted redox homeostasis by altering antioxidant enzyme activities and osmolyte accumulation, and induced pronounced shifts in rhizosphere microbial community composition and structure. Microbial inoculation modified osmolyte accumulation and antioxidant enzyme activity, with effects varying in magnitude and direction across moisture regimes and physiological indicators, and was generally associated with elevated soil nutrient availability and extracellular enzyme activities, though the magnitude of these effects was modulated by moisture regime. The MIX treatment showed the strongest positive responses for a subset of plant physiological and soil biochemical indicators. Bacterial and fungal co-occurrence networks exhibited divergent structural responses to inoculation and drought, with network complexity (e.g., connectivity and modularity) differentially shaped by both factors. Mantel tests and partial least squares path modeling revealed that under well-watered and moderate drought conditions, soil physicochemical properties were the primary drivers of microbial alpha and beta diversity; in contrast, under severe drought, osmotic adjustment emerged as the dominant physiological determinant of biomass maintenance. Collectively, these results reveal that the functional outcomes of microbial inoculation are contingent upon abiotic context, particularly drought intensity. This study provides a mechanistic basis and preliminary empirical evidence for subsequent field validation and further development of moisture-matched microbial intervention strategies for the ecological restoration of drought-vulnerable *A. splendens* ecosystems.

Keywords: *Achnatherum splendens*; drought intensity; *Bacillus subtilis*; *Rhizophagus irregularis*; co-occurrence network

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1. Introduction

Drought constrains plant establishment by reducing water availability, nutrient uptake, and photosynthetic capacity, and climate change is expected to increase both the extent and severity of this stress [1]. Enhancing drought tolerance is therefore a key objective for vegetation restoration and land management in arid regions. *Achnatherum splendens* is a perennial xerophytic grass species native to and widely distributed in deserts, semideserts, and saline–alkaline grasslands of northwestern China [2]. Its extensive root system and tolerance to nutrient-poor, saline, and alkaline soils make it valuable for soil and water conservation, wind erosion control, sand stabilization, and restoration of degraded grasslands [3]. The species can also improve soil nutrient availability, enhance sandy-soil structure, and contribute to community stability [4]. Severe or prolonged drought, however, inhibits germination and biomass accumulation, reduces root water uptake, and degrades the rhizosphere micro-environment through reductions in soil enzyme activity and microbial diversity [5]. These constraints collectively limit the large-scale establishment of *A. splendens* on degraded desert lands.

Beneficial rhizosphere microorganisms can enhance plant stress tolerance, nutrient acquisition, and soil quality [6]. *Bacillus subtilis* promotes nutrient availability through processes such as nitrogen fixation, phosphorus solubilization, potassium mobilization, and phytohormone production. It may also improve abiotic-stress tolerance by inducing systemic resistance, releasing volatile and nonvolatile metabolites, and modulating rhizosphere microbial communities [7,8]. Arbuscular mycorrhizal fungi form symbiotic associations with approximately 80% of terrestrial plant species [9,10]. Their extraradical hyphae expand the volume of soil explored by roots; facilitate water and nutrient uptake; and can modify osmotic adjustment, antioxidant defenses, and rhizosphere community structure [11,12]. Although the drought-mitigating effects of BS and AMF have been separately demonstrated in several crops, it remains unclear whether their combined effects on *A. splendens* are complementary, additive, or context-dependent.

The system comprises three interacting components: the host plant, rhizosphere soil, and the associated bacterial and fungal communities. Drought acts as an environmental filter across all three components, while inoculation can alter both plant physiology and microbial interactions. Most previous studies have focused on crop performance and physiological indicators rather than the integrated responses of plant traits, soil biochemical processes, microbial diversity, and community networks. We therefore conducted a controlled pot experiment across three moisture regimes and four inoculation treatments. We addressed the following questions: (1) Do BS and AMF, applied separately or in combination, improve the physiological responses of *A. splendens* to drought? (2) Do bacterial and fungal diversity and community structure show consistent responses to inoculation across different drought intensities? (3) How do the soil-microbe–plant pathways associated with biomass change as drought intensity increases? By explicitly examining these interconnected components, our study evaluates whether inoculant performance is contingent on environmental context and provides a foundation for moisture-specific microbial management in dryland restoration.

2. Material and Methods

2.1. Experimental Materials

Achnatherum splendens seeds were collected in Ordos, Inner Mongolia, China, in 2025. Seeds were washed with running tap water to remove surface dust, immersed in 75% ethanol for 60 s, then transferred into 5% sodium hypochlorite solution supplemented with 2–3 drops of Tween-80 for 8 min, followed by five rinses with sterile deionized water to remove residual disinfectant. After surface sterilization, seeds were sown in nursery trays

containing commercial potting soil. Seedlings were maintained in an experimental greenhouse and watered regularly to keep the substrate moist for subsequent transplantation.

The microbial inoculants used in this study included *Bacillus subtilis* and arbuscular mycorrhizal fungus *Rhizophagus irregularis*, with detailed strain information provided for experimental reproducibility. The *Bacillus subtilis strain* (Strain ID: GT041-KC011) was purchased from the National Center for Research and Extension of Microbial Fertilizer Technology (China). The commercial microbial preparation had a valid viable bacterial concentration of approximately 1×10^{11} colony-forming units (CFUs) g^{-1}, with consistent strain purity and activity guaranteed by the supplier. The *Rhizophagus irregularis* strain (Strain ID: BGC AH01) was preserved and propagated in our laboratory. To obtain sufficient AMF inoculum, a 1:1 (v/v) mixture of sand and zeolite was high-temperature sterilized and used as the culture substrate, with maize (*Zea mays*) selected as the host plant for strain propagation. After a 120-day propagation cycle, the substrate mixture containing abundant fungal spores, extraradical hyphae, and colonized maize root fragments was harvested and served as the standardized *Rhizophagus irregularis* inoculum for the formal experiment. Furthermore, *Bacillus subtilis* and *Rhizophagus irregularis* have been proven to have the functional characteristics of alleviating drought.

Commercial organic nutrient potting soil was purchased from Sanyuan Herui Biotechnology Co., Ltd., Binzhou City, China. This general horticultural substrate consists of turf soil, perlite, and fermented crop straw, with organic matter $\geq$45%, total NPK $\geq$ 6%, and pH of 5.5–6.5. The plant growth substrate was prepared by mixing this commercial potting soil with quartz sand at a volume ratio of 1:2. After thorough homogenization and wetting, the mixed substrate was packaged into 500 custom sterilization bags and sterilized using a GXMQ-2.5 medium sterilizer (Shanghai Shenan Medical Instrument Co., Ltd., Shanghai, China). The full automated sterilization program took ~7 h (including preheating, staged heating, 90 min holding at 121 °C/0.14 MPa, and natural cooling). The staged heating profile included 30 min at 105 °C (0.080 MPa) and 5 min at 115 °C (0.120 MPa), followed by the core sterilization stage of 90 min at 121 °C (0.140 MPa) and a subsequent 30 min holding stage. The sterilized substrate was spread evenly in an ultra-clean bench and air-dried for 1 day to stabilize moisture and temperature before use.

2.2. Experimental Design

A two-factor factorial experiment was conducted with inoculation and soil moisture as the factors. The four inoculation treatments were CK (sterilized preparations of both inoculants), BS (live *B. subtilis* plus sterilized AMF inoculum), AMF (live AMF plus sterilized *B. subtilis* preparation), and MIX (live *B. subtilis* plus live AMF). The three moisture regimes were normal moisture (NM; 70 $\pm$ 5% of maximum water-holding capacity), moderate drought (MD; 50 $\pm$ 5%), and severe drought (SD; 30 $\pm$ 5%). The experiment comprised 12 treatment combinations (Table 1), each with four replicates, for a total of 48 pots. Within a single pot, subsamples were collected from multiple individual plants. For plant physiological measurements, leaf tissues from several plants within one pot were mixed and homogenized to generate a composite sample. Rhizosphere soil was harvested from the root surfaces of multiple plants in each pot; these rhizosphere subsamples from the same pot were pooled and thoroughly homogenized to yield one composite rhizosphere soil sample per pot. All plant and soil variables were measured on these composite samples, so each pot contributed a single data point for statistical analysis.

The experiment began in an experimental greenhouse in July 2025. When seedlings had developed three to four true leaves, individuals of similar size were transplanted. Each pot received 2 kg of sterilized substrate and seven seedlings. The *B. subtilis* inoculant was a solid powder formulation with a viable concentration of 1×10^{11} CFU g^{-1}. For BS

and MIX treatments, 1.4 g of live *B. subtilis* powder was applied per pot, corresponding to 0.07% of the 2 kg substrate mass and falling within the target application range of 0.05–0.10%. To achieve uniform distribution, the bacterial powder was first premixed with 100 g sterilized substrate, then thoroughly blended with the remaining substrate. The *Rhizophagus irregularis* inoculum was a mixture of sand, zeolite, fungal spores, extraradical hyphae, and colonized maize root fragments harvested after 120 days of propagation using maize as the host. The inoculum contained 35–40 spores g^{-1} dry inoculum, with maize root fragments showing >70% AMF colonization. For AMF and MIX treatments, 200 g of this live AMF inoculum was mixed into each pot's substrate at an inoculum: substrate ratio of 1:10. CK pots received equivalent amounts of autoclaved *B. subtilis* powder and autoclaved AMF inoculum matrix to balance organic carriers, sand, zeolite and root fragments across all treatments. All pots were maintained under normal moisture (70 ± 5% WHC) for 2 months after transplanting to allow root colonization and the establishment of microbial inoculants. During this pre-culture period, soil moisture was checked regularly. After the two-month establishment phase, drought treatments were imposed. Soil water content was maintained gravimetrically: each pot was weighed daily at 17:00, and deionized water was added to restore the target moisture level. This drought phase lasted for 30 days. After this period, plant and soil samples were harvested for analysis. The core objective of the present study was to investigate the overall effects of exogenous *Rhizophagus irregularis* and *Bacillus subtilis* co-inoculation on plant growth performance and soil microbial community characteristics under drought gradients rather than to explore the symbiosis formation process or quantify root colonization intensity.

Table 1. Experimental treatments.

Inoculation/Moisture	Normal Moisture (NM)	Moderate Drought (MD)	Severe Drought (SD)
CK (two sterilized inocula)	CK	CK	CK
BS (*B. subtilis* + sterilized AMF)	BS	BS	BS
AMF (AMF + sterilized *B. subtilis*)	AMF	AMF	AMF
MIX (AMF + *B. subtilis*)	MIX	MIX	MIX

2.3. Biomass Measurement and Plant Physiological Traits

At the end of the experiment, plants were harvested and washed. Samples were placed in paper envelopes and heated at 105 °C for 30 min to halt metabolic activity, then dried at 65 °C to a constant mass. Aboveground biomass (AGB) and belowground biomass (BGB) were measured separately [13], and total biomass (TB) was calculated as their sum.

Fresh *A. splendens* leaves were collected, wrapped in aluminum foil, immediately frozen in liquid nitrogen, and stored at −80 °C. Proline (Pro), soluble protein (SP), soluble sugar (SS), and malondialdehyde (MDA) concentrations, together with superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities, were measured using commercial assay kits (Solarbio, Beijing, China) according to the manufacturer's protocols. Leaf relative chlorophyll content was determined using a SPAD-502 Plus chlorophyll meter (Konica Minolta, Chiyoda City, Japan). For each replicate, six fully expanded intact leaves of *Achnatherum splendens* were selected. Six SPAD readings were recorded on each leaf while avoiding leaf midribs, and the average value per leaf was used for subsequent statistical analysis. All measurements were conducted between 9:00 and 11:00 a.m. to reduce the influence of diurnal changes in leaf physiological status.

2.4. Soil Physicochemical Properties and Enzyme Activities

At the end of the experiment, rhizosphere soil was collected, cleared of plant debris and stones, sealed in bags, and transported to the laboratory. Samples were air-dried, ground, and sieved for physicochemical and enzyme analyses. Soil pH was measured potentiometrically with a glass-electrode pH meter at a water-to-soil ratio of 2.5:1 [14]. Soil total carbon (S-TC) was determined using a total organic carbon analyzer (SSM-5000A) [15], soil total nitrogen (S-TN) by the Kjeldahl method [16], and soil total phosphorus (S-TP) by alkaline fusion followed by molybdenum–antimony colorimetry [17].

2.5. Soil Microbial Community Analysis

Microbial DNA was extracted from 0.5 g of fresh soil using a PowerSoil DNA Isolation Kit (Qiagen, formerly MoBio, San Diego, CA, USA), and DNA purity and integrity were assessed using 1% agarose-gel electrophoresis. The V3–V4 region of the bacterial 16S rRNA gene was amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) [18]. The fungal ITS region was amplified with primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGTGTTCTTCATCGATGC-3′) [19]. PCR cycling conditions were as follows: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 5 min. Amplicons were quantified, pooled, and used for library preparation, followed by paired-end sequencing on an Illumina MiSeq PE300 platform (Shanghai Majorbio Biopharm Technology Co., Ltd., Shanghai, China). Raw paired-end reads were merged and quality-filtered with a threshold of Q20, and chimeric sequences were removed to generate clean reads. Amplicon sequence variants (ASVs) were inferred using the DADA2 pipeline rather than traditional operational taxonomic unit (OTU) clustering. For bacterial 16S rRNA sequencing, a total of 34,298,824 raw reads were obtained; after quality filtering, denoising and chimera removal via the DADA2 pipeline, 3,194,455 high-quality reads were retained, yielding 12,813 bacterial ASVs. For fungal ITS sequencing, 3,097,986 raw reads were processed, and 2,686,299 high-quality reads were retained after DADA2, yielding 1940 fungal ASVs. Taxonomic assignment of bacterial 16S rRNA ASVs was performed against the SILVA v138 reference database. Fungal ITS ASVs were taxonomically annotated using the UNITE database (version 9.0). To reduce bias from uneven sequencing depth, the ASV feature table was rarefied to the minimum sequencing depth across all samples. Bacterial and fungal alpha-diversity indices (Chao1 and Shannon) were calculated independently based on the rarefied ASV table.

2.6. Statistical Analysis

Data were organized in Microsoft Excel 2019 and analyzed using SPSS 26.0 and R 4.2.2. Normality and homogeneity of variance were assessed prior to analysis; variables that did not meet these assumptions were log-transformed. One-way ANOVA followed by Fisher's least significant difference test was used for pairwise comparisons within each drought level, and two-way ANOVA was used to test the main and interactive effects of drought and microbial inoculation. Statistical significance was set at $p < 0.05$. Microbial co-occurrence networks were constructed separately for each treatment ($n = 4$ biological replicates). Pairwise Spearman correlations between ASVs were calculated, and only edges with $|r| > 0.6$ and FDR-adjusted $p < 0.01$ were retained to reduce spurious correlations. Topological properties of co-occurrence networks were calculated using the R igraph package, and networks were visualized in Gephi version 0.9.2. Network complexity and robustness were calculated following Yuan et al. [20]. Mantel tests were run per drought gradient (n = 16, 999 permutations). Partial least-squares path models (PLS-PMs) were fitted separately for

each drought gradient using the plspm package. Each PLS-PM included 16 independent samples (4 inoculation treatments × 4 biological replicates per treatment) to examine correlational associations among soil properties, microbial communities, plant physiology, and plant biomass. All figures were visualized using ggplot2 in R or Origin 2022.

3. Results

3.1. Effects of Microbial Inoculation on Physiological Traits of Achnatherum splendens Under Drought Stress

Aboveground and belowground biomass declined significantly with increasing drought intensity; however, inoculation with BS, AMF, or their mixture (MIX) generally mitigated biomass loss (Figure 1). Under non-stressed (NM) conditions, aboveground biomass of BS and MIX was significantly greater than CK ($p < 0.05$), whereas AMF showed slightly higher aboveground biomass without statistical significance. Under moderate drought (MD) and severe drought (SD), none of the three inoculation treatments yielded significantly higher aboveground biomass compared with CK. Proline, soluble protein, and soluble sugar concentrations varied with drought severity; MIX did not consistently maintain the highest proline concentration. Notably, soluble sugar values for MIX were numerically lower than CK across all three moisture regimes, with a significant difference only under MD. Inoculation altered osmoprotectant concentrations relative to CK, though statistical significance depended on both inoculant treatment and moisture condition. Drought severity did not significantly alter MDA levels; within each moisture regime, MDA concentrations tended to be higher in inoculated plants than in CK, with significant differences detected for particular inoculant treatments ($p < 0.05$). Changes in SOD, POD, and CAT activities were not progressive with increasing drought intensity. Inoculation modulated antioxidant enzyme activities relative to CK, but significant differences were conditional upon moisture level and inoculant type, and MIX did not exhibit the highest enzyme activity in most conditions.

Two-way ANOVA revealed significant main effects of drought and inoculation on aboveground biomass, proline concentration, and SOD activity ($p < 0.05$; Table 2), while their interaction was not significant for these three indicators. Significant interactive effects between drought and inoculation were observed for POD and CAT activities ($p < 0.05$; Table 2). We hypothesize that inoculation may activate plant osmoprotective and antioxidant systems, which could partly contribute to biomass maintenance under drought stress. The elevated MDA observed in some inoculation treatments might reflect accelerated lipid turnover associated with plant-microbe symbiotic signaling rather than irreversible oxidative membrane damage.

Plant total carbon (P-TC) and total nitrogen (P-TN) exhibited no consistent trend across drought severity levels, and neither drought severity nor microbial inoculation significantly affected plant carbon or nitrogen accumulation (Figure 2). No significant differences in P-TC or P-TN were observed among the control (CK) and inoculation treatments (BS, AMF, and MIX) within any moisture regime. In contrast, chlorophyll content declined significantly with increasing drought severity and was significantly higher under NM than under MD or SD ($p < 0.05$). BS inoculation significantly enhanced chlorophyll content relative to CK across all three moisture regimes ($p < 0.05$), whereas AMF inoculation exerted no statistically significant effect. Two-way ANOVA revealed a highly significant main effect of drought on chlorophyll content ($p < 0.001$) and a significant drought × inoculation interaction ($p < 0.05$) but no significant main effects on or interactions with P-TC or P-TN. Notably, the MIX treatment resulted in significantly lower chlorophyll content than CK, BS, and AMF across all moisture regimes, demonstrating a consistent suppressive effect on leaf chlorophyll accumulation induced by co-inoculation of *B. subtilis* and AMF. We hypothesize that this

reduction may indicate a potential antagonistic interaction between *B. subtilis* and AMF under coinoculation conditions. This observation appears decoupled from the relatively better growth performance, osmotic adjustment, and antioxidant capacity observed in MIX plants, which may suggest a possible trade-off between microbe-enhanced stress tolerance and leaf photosynthetic pigment synthesis; however, this interpretation remains speculative and requires further experimental validation. Two-way ANOVA revealed a highly significant main effect of drought on chlorophyll content ($p < 0.001$) and a significant drought × inoculation interaction ($p < 0.05$) but no significant main effects on or interactions with P-TC or P-TN.

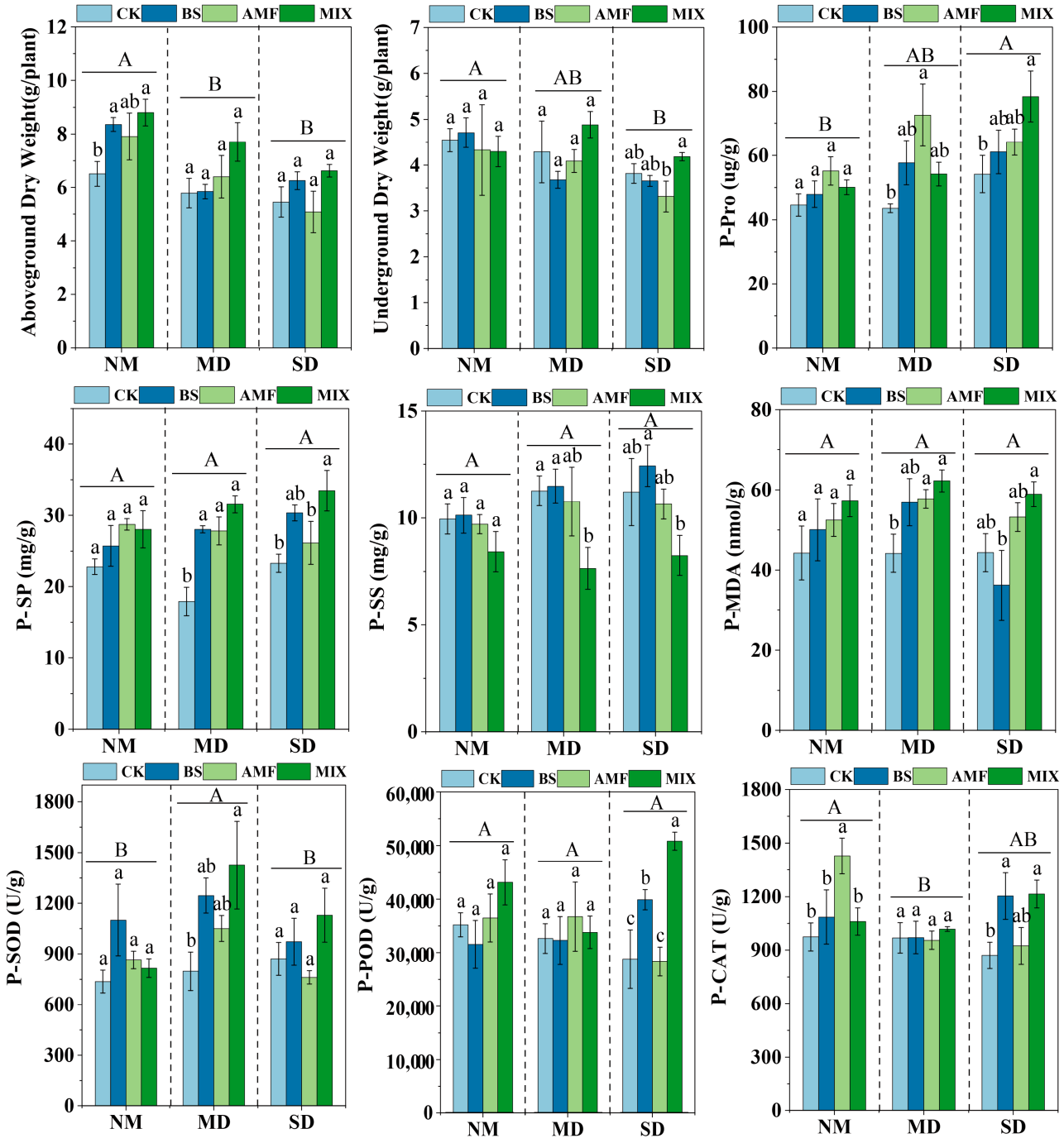


Figure 1. Growth and physiological traits of *Achnatherum splendens* under different treatments. Note: Different lowercase letters indicate differences among inoculation treatments within the same moisture regime, and different uppercase letters indicate differences among moisture regimes

($p < 0.05$). P-Pro plant proline; P-SP, plant soluble protein; P-SS, plant soluble sugar; P-MDA, plant malondialdehyde; P-SOD, plant superoxide dismutase; P-POD, plant peroxidase; P-CAT, plant catalase. CK, sterilized preparations of both inoculants; BS, live *B. subtilis* plus sterilized AMF inoculum; AMF, live AMF plus sterilized *B. subtilis* preparation and MIX live *B. subtilis* plus live AMF. NM, Normal Moisture; MD, Moderate Drought; SD, Severe Drought. The same lettering convention applies below.

Table 2. Two-way ANOVA for effects of drought and microbial inoculation on plant physiological-growth traits.

Indicators	Moisture			Inoculation			Moisture × Inoculation		
	df	F	*p*	df	F	*p*	df	F	*p*
Aboveground Dry Weight	2	13.62	**<0.001**	3	5.23	**0.004**	6	0.89	0.511
Underground Dry Weight	2	3.21	0.052	3	0.99	0.410	6	0.71	0.630
P-Pro	2	7.37	**0.002**	3	5.14	**0.005**	6	1.79	0.128
p-SP	2	1.37	0.257	3	12.90	**<0.001**	6	1.59	0.180
P-SS	2	1.26	0.295	3	6.26	**0.002**	6	0.40	0.872
P-MDA	2	1.88	0.168	3	5.09	**0.005**	6	0.92	0.495
P-SOD	2	4.02	**0.027**	3	4.39	**0.010**	6	1.31	0.280
P-POD	2	0.75	0.479	3	4.20	**0.012**	6	2.51	**0.039**
P-CAT	2	2.94	0.065	3	2.19	0.106	6	3.20	**0.013**

Note: Bold values denote significance at $p < 0.05$.

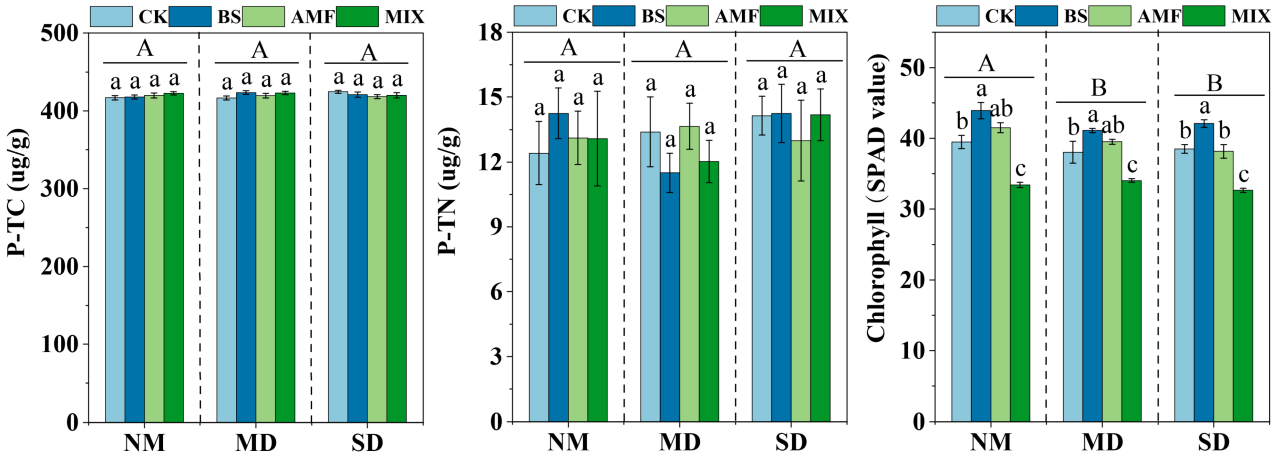


Figure 2. Nutrient and chlorophyll contents of *Achnatherum splendens* under different treatments. Note: Different lowercase letters indicate differences among inoculation treatments within the same moisture regime, and different uppercase letters indicate differences among moisture regimes ($p < 0.05$). P-TC, plant total carbon; P-TN, plant total nitrogen. CK, sterilized preparations of both inoculants; BS, live *B. subtilis* plus sterilized AMF inoculum; AMF, live AMF plus sterilized *B. subtilis* preparation and MIX live *B. subtilis* plus live AMF. NM, Normal Moisture; MD, Moderate Drought; SD, Severe Drought.

3.2. Effects of Microbial Inoculation on Soil Nutrients and Enzyme Activities Under Drought Stress

Soil pH showed no consistent trend across drought severity levels, with significant differences among inoculation treatments detected under NM and MD ($p < 0.05$). S-TC, S-TN and S-TP were not significantly affected by drought intensity, and inoculation effects were variable rather than uniformly higher across all inoculant treatments relative to CK. Two-way ANOVA revealed no significant main effects of inoculation or drought stress on soil pH, S-TC, or S-TP ($p > 0.05$). Soil enzyme activities exhibited divergent responses to increasing drought severity: S-UE and S-β-GC generally rose as drought intensified, while S-ALP and S-POD did not show a consistent increase across drought gradients.

Inoculation enhanced soil enzyme activities, but significant differences from CK depended on moisture level and inoculant type rather than occurring for all three inoculants under every moisture regime. MIX produced the highest enzyme activities in most cases. Two-way ANOVA showed significant main effects of drought and inoculation on S-UE and S-β-GC, and S-β-GC exhibited a significant drought × inoculation interaction ($p < 0.05$; Figure 3; Table 3).

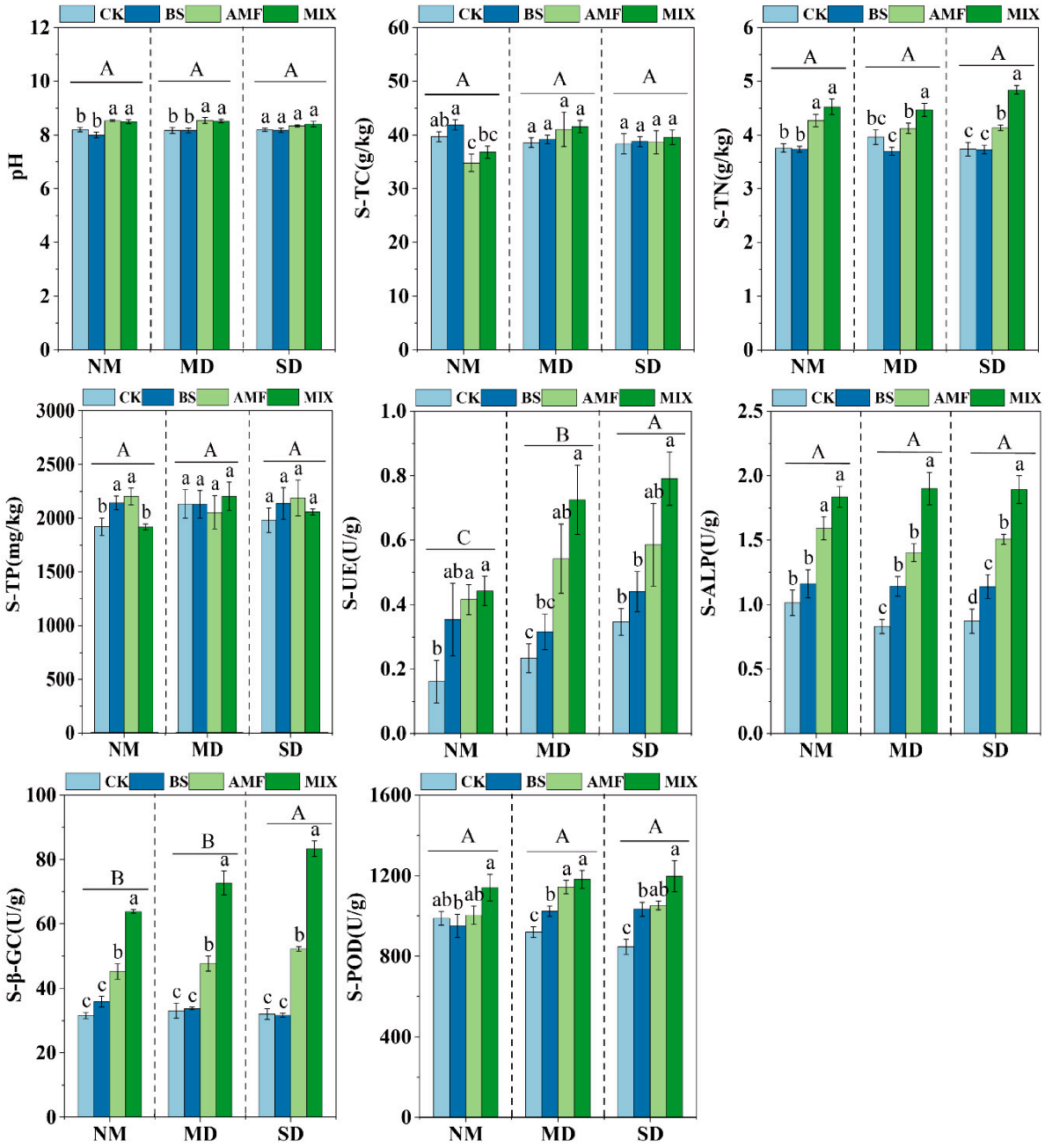


Figure 3. Soil nutrient contents and enzyme activities under different treatments. Note: Different lowercase letters indicate differences among inoculation treatments within the same moisture regime, and different uppercase letters indicate differences among moisture regimes ($p < 0.05$). S-TC, soil total carbon; S-TN, soil total nitrogen; S-TP, soil total phosphorus; S-UE, soil urease; S-ALP, soil alkaline phosphatase; S-β-GC, soil β-glucosidase; S-POD, soil peroxidase. CK, sterilized preparations of both inoculants; BS, live *B. subtilis* plus sterilized AMF inoculum; AMF, live AMF plus sterilized *B. subtilis* preparation and MIX live *B. subtilis* plus live AMF. NM, Normal Moisture; MD, Moderate Drought; SD, Severe Drought.

Table 3. Two-way ANOVA results showing main and interactive effects of drought stress and microbial inoculation on soil extracellular enzyme activities.

Indicator	Moisture			Inoculation			Moisture × Inoculation		
	df	F	*p*	df	F	*p*	df	F	*p*
pH	2	0.72	0.492	3	16.10	**<0.001**	6	1.11	0.378
S-TC	2	1.40	0.259	3	0.69	0.562	6	2.13	0.074
S-TN	2	0.25	0.779	3	47.44	**<0.001**	6	1.97	0.097
S-TP	2	0.53	0.591	3	0.96	0.425	6	0.82	0.565
S-UE	2	6.02	**0.006**	3	14.22	**<0.001**	6	0.80	0.576
S-ALP	2	0.86	0.432	3	67.78	**<0.001**	6	0.54	0.775
S-β-GC	2	8.80	**<0.001**	3	292.93	**<0.001**	6	7.19	**<0.001**
S-POD	2	1.17	0.323	3	17.12	**<0.001**	6	1.75	0.137

Note: Bold values denote significance at $p < 0.05$.

3.3. Effects of Microbial Inoculation on Soil Microbial Communities Under Drought Stress

Bacterial Chao1 richness increased from non-stressed (NM) to moderate drought (MD), but SD did not differ significantly from either NM or MD (Figure 4a). Within each drought level, inoculation increased bacterial Chao1 relative to CK, but significant differences were only detected for selected inoculant treatments rather than uniformly across BS, AMF and MIX. MIX generally produced the highest Chao1 values. The bacterial Shannon index was lower under NM compared with MD and SD. Inoculation tended to elevate bacterial Shannon diversity relative to CK; however, significant differences among inoculation treatments were observed only under NM, and no significant inoculation-related differences were found under MD or SD (Figure 4b). Fungal Chao1 and Shannon indices showed no significant variation across drought intensities, and no significant differences among inoculation treatments were detected within any moisture regime (Figure 4c,d). Principal coordinates analysis (PCOA) based on Bray–Curtis dissimilarity illustrated shifts in bacterial and fungal community composition. Community dissimilarity was assessed using ANOSIM, with $R = 0.263$ and $p = 0.001$ for bacteria and $R = 0.076$ and $p = 0.011$ for fungi (Figure 4e,f).

LEfSe analysis (LDA score > 2.0; $p < 0.05$) identified differentially abundant bacterial phyla including *Acidobacteriota*, *Bacillota*, *Gemmatimonadota*, and *RCP2-54*, which exhibited treatment-specific enrichment patterns (Figure 4g). At the genus level, *Acinetobacter*, *Agromyces*, *Aminobacter*, and *Ammoniphilus* were among the taxa with significant differential abundance across treatments. For fungi, *Glomeromycota* was among the phyla showing significant treatment-associated enrichment; differentially abundant fungal genera included *Botryotrichum*, *Cephalotrichum*, *Claroideoglomus*, and *Gibellulopsis* (Figure 4h).

Drought severity and microbial inoculation jointly shaped the size of bacterial co-occurrence networks and the proportion of positive correlations (Figure 5). Under NM, the MIX treatment formed the highest edge count (523 edges), far exceeding CK (263), BS (315), and AMF (288). MIX also maintained the highest edge count under MD and SD, while CK had the smallest network in each moisture regime. For CK and AMF, edge numbers rose with increasing drought intensity; edge count for BS remained unchanged (315 edges) between NM and MD before increasing to 329 edges under SD.

The proportion of positive correlations displayed divergent responses across treatments and drought levels: CK had the highest proportion of positive edges under NM (59.70%), BS under MD (60.83%), and AMF under SD (55.39%). Accordingly, the treatment with the largest co-occurrence network did not consistently show the highest proportion of positive correlations.

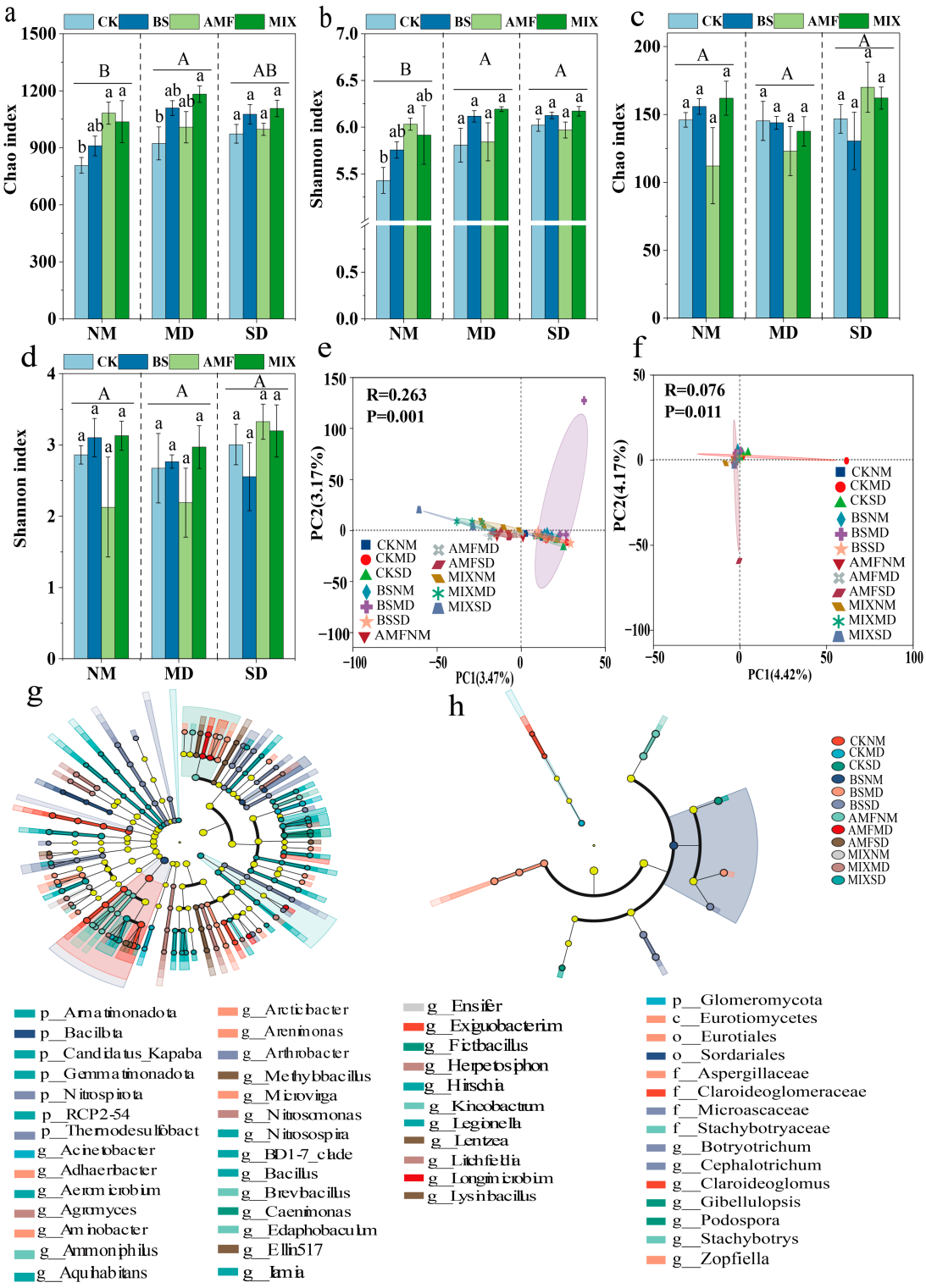


Figure 4. Soil microbial community characteristics under different treatments. Note: Different lowercase letters indicate differences among inoculation treatments within the same moisture regime, and different uppercase letters indicate differences among moisture regimes ($p < 0.05$). (**a**) Bacterial Chao1 index; (**b**) bacterial Shannon index; (**c**) fungal Chao1 index; (**d**) fungal Shannon index; (**e**) bacterial PCoA; (**f**) fungal PCoA; (**g**) bacterial LEfSe analysis; (**h**) fungal LEfSe analysis. CK, sterilized preparations of both inoculants; BS, live *B. subtilis* plus sterilized AMF inoculum; AMF, live AMF plus sterilized *B. subtilis* preparation and MIX live *B. subtilis* plus live AMF. NM, Normal Moisture; MD, Moderate Drought; SD, Severe Drought. *Zi-Pi* soil bacterial network node analysis (Figure 6). For all treatment bacterial communities, the peripheral nodes were the dominant groups, while the connecting nodes

were the core groups that maintained the connectivity of the modules. The number of module hubs was scarce, and there were no typical network hub species. Under the same drought stress conditions, the number of connecting nodes and *Pi* values in the BS, AMF, and MIX treatments were all higher than those in the CK. MIX could enrich more connecting nodes and module hubs. As the drought stress intensified, the overall interaction intensity of the connecting nodes in each group gradually increased. In severe drought conditions, the enrichment effect of key groups in the *Zi* treatment was more prominent. Drought and inoculation treatments resulted in differences in the number of network key nodes and the *Zi* and *Pi* indices. The interaction between the two significantly changed the interaction structure of microbial community modules.

Fungal co-occurrence network responses diverged markedly from those observed in bacterial communities (Figure 7). Under NM, AMF inoculation generated the highest edge count (395 edges), substantially exceeding those of CK (309), BS (272), and MIX (227). Under moderate drought (MD), CK exhibited the highest edge count (436), followed by MIX (276), BS (275), and AMF (258). Under severe drought (SD), BS yielded the greatest number of edges (337), while MIX had the lowest edge count (224). The proportion of positive correlations reached 97.97% in the NM-AMF network and dropped sharply to 70.48% in NM-MIX. Under MD, positive correlations exceeded 90% across all treatments and peaked in CK (95.64%). Responses of positive edge proportions to severe drought were treatment-dependent rather than uniformly declining; MIX had the lowest proportion of positive edges under SD (84.82%). Collectively, these correlation-based structural patterns suggest that AMF fosters a densely connected fungal community under optimal moisture conditions, whereas BS enhances fungal connectivity under severe drought stress; MIX, however, increased bacterial network size without concomitant expansion of the fungal network.

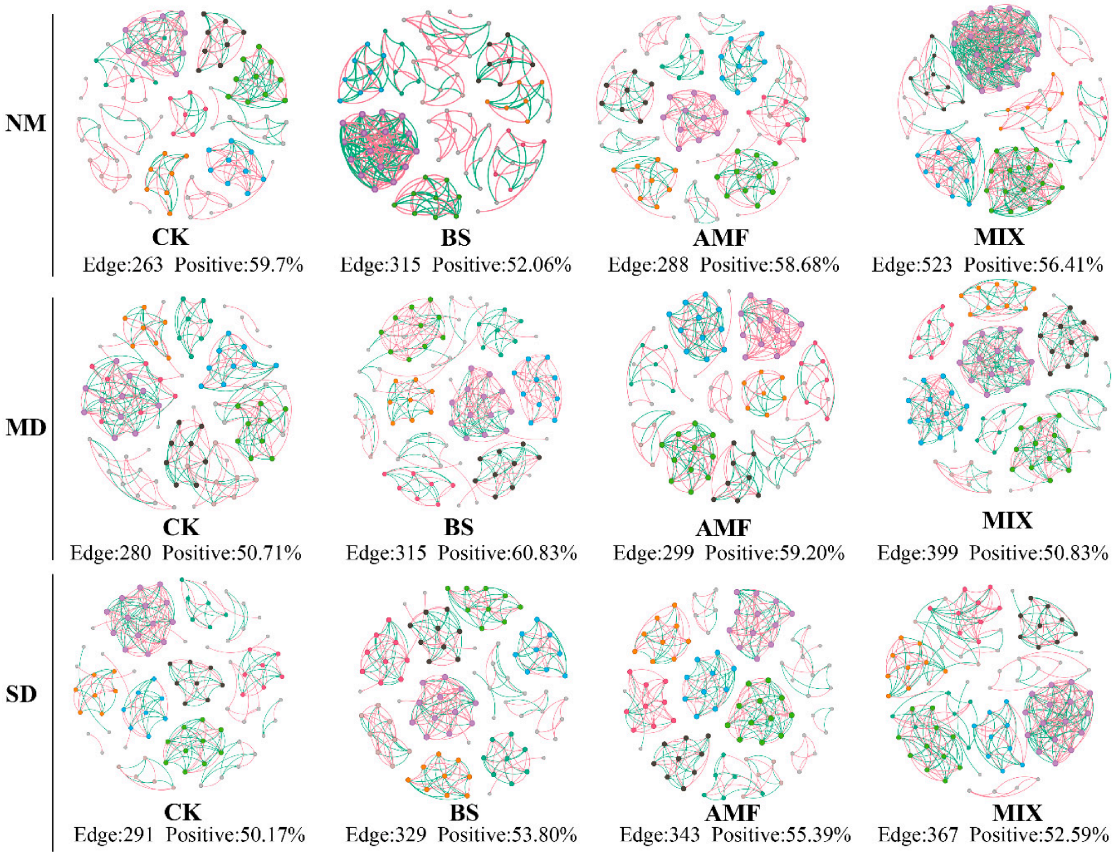


Figure 5. Co-occurrence network analysis of soil bacterial communities. Note: Node size is proportional to degree (number of connected nodes). Red edges indicate positive associations, and green

edges indicate negative associations. Nodes are colored by taxonomic group. CK, sterilized preparations of both inoculants; BS, live *B. subtilis* plus sterilized AMF inoculum; AMF, live AMF plus sterilized *B. subtilis* preparation and MIX live *B. subtilis* plus live AMF. NM, Normal Moisture; MD, Moderate Drought; SD, Severe Drought.

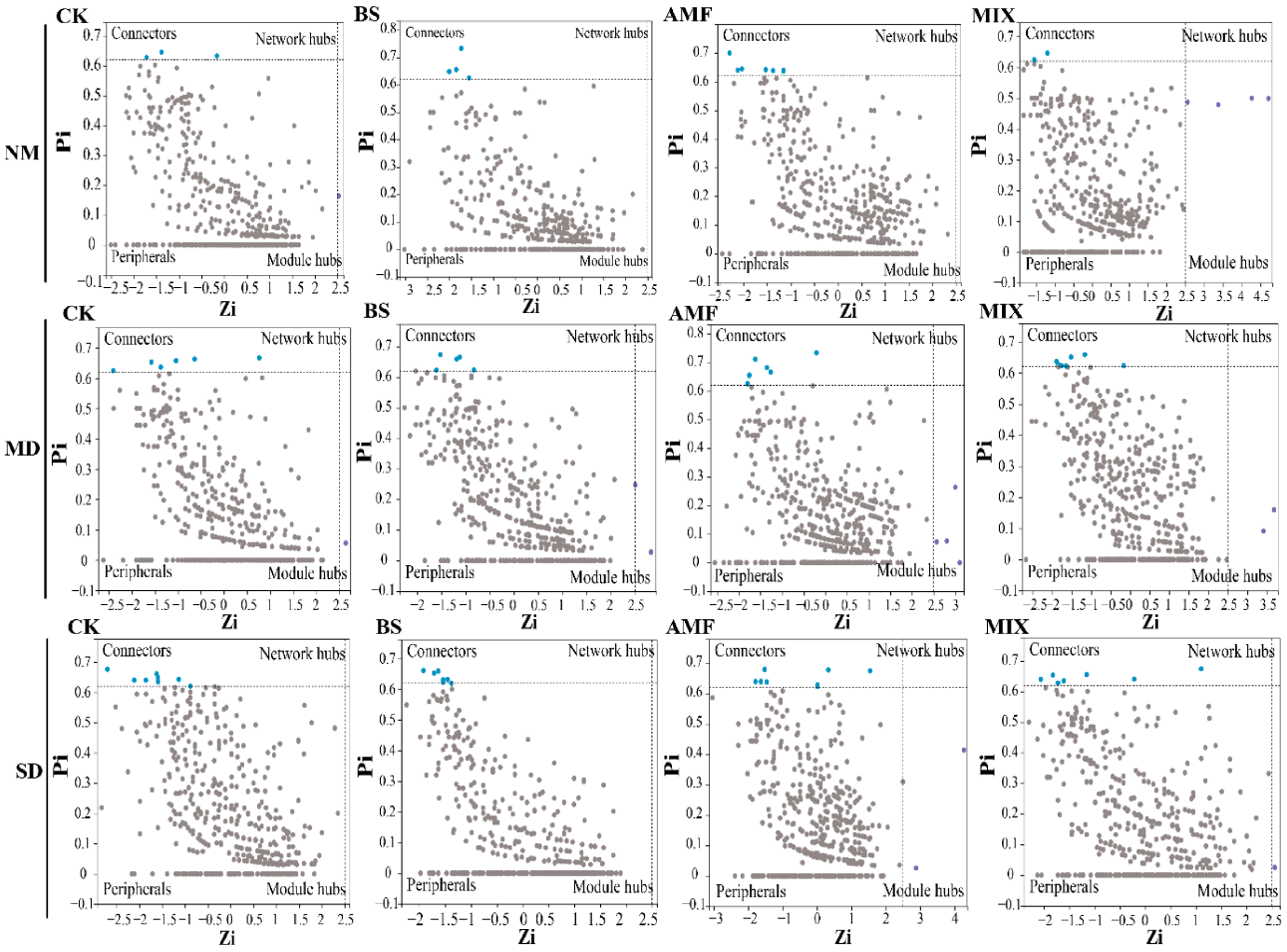


Figure 6. Within-module connectivity (*Zi*) and among-module connectivity (*Pi*) of soil bacterial networks. Note: Nodes represent taxa or functions and are classified by topology into four categories: module hubs (highly connected within modules; $Zi > 2.5$ and $Pi < 0.65$), connectors (high connectivity among modules; $Zi < 2.5$ and $Pi > 0.65$), network hubs (high connectivity both within and among modules; $Zi > 2.5$ and $Pi > 0.65$), and peripherals (low connectivity within and among modules; $Zi < 2.5$ and $Pi < 0.65$). The dashed lines indicate the thresholds of Zi = 2.5 and Pi = 0.65. All categories except peripherals are considered keystone nodes. CK, sterilized preparations of both inoculants; BS, live *B. subtilis* plus sterilized AMF inoculum; AMF, live AMF plus sterilized *B. subtilis* preparation and MIX live *B. subtilis* plus live AMF. NM, Normal Moisture; MD, Moderate Drought; SD, Severe Drought.

Zi-Pi analysis of fungal co-occurrence networks (Figure 8) revealed that peripheral nodes overwhelmingly predominated across all treatments. Connector taxa bridging distinct modules were sparse; module hubs were uncommon, and no network hubs (i.e., taxa exhibiting both high within-module connectivity and high among-module connectivity) were detected. This topological pattern points to potential constraints on within-module specialization and limited inter-modular connectivity.

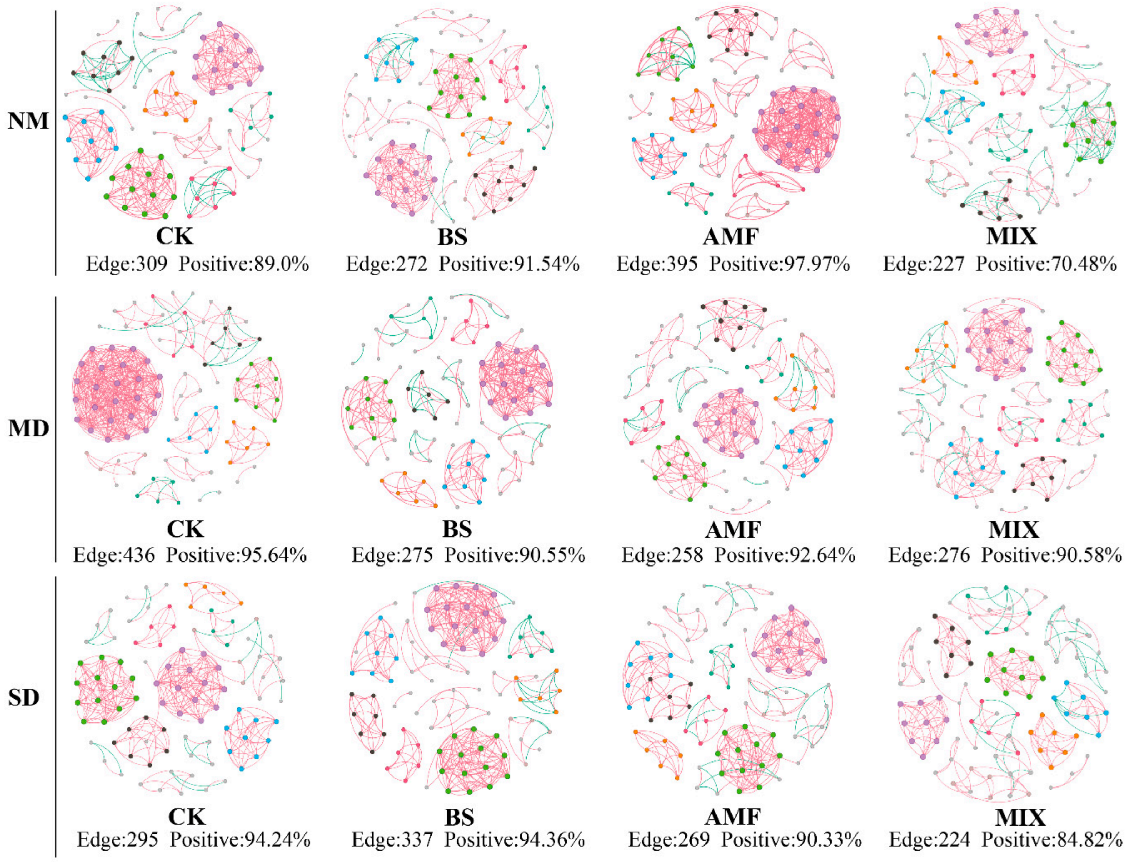


Figure 7. Co-occurrence network analysis of soil fungal communities. Note: Node size is proportional to degree (number of connected nodes). Red edges indicate positive associations, and green edges indicate negative associations. Nodes are colored by taxonomic group. CK, sterilized preparations of both inoculants; BS, live *B. subtilis* plus sterilized AMF inoculum; AMF, live AMF plus sterilized *B. subtilis* preparation and MIX live *B. subtilis* plus live AMF. NM, Normal Moisture; MD, Moderate Drought; SD, Severe Drought.

3.4. Soil–Microbe–Plant Interactions

Through Mantel analysis, it was found (Figure 9) that under normal moisture (NM) conditions, bacterial diversity was significantly positively correlated with β-glucosidase and soluble proteins ($p < 0.05$), and fungal diversity was significantly positively correlated with underground biomass and proline ($p < 0.05$). Under moderate drought (MD) conditions, bacterial diversity was significantly positively correlated with plant carbon ($p < 0.05$), and fungal diversity was significantly positively correlated with plant peroxidase ($p < 0.05$). Under severe drought (SD) conditions, environmental factors had no significant effect on bacterial and fungal diversity, indicating that soil bacterial and fungal diversity was affected by multiple factors together.

Further, through the structural equation model, the multi-level regulatory pathways of soil environment–microbial community–plant physiological growth under different drought gradients were revealed (Figure 9). Under normal moisture (NM) conditions, soil pH significantly positively drove soil enzyme activity and bacterial community diversity, bacterial diversity significantly enhanced plant nutrient accumulation and the synthesis of osmotic regulatory substances, soil nutrients were significantly positively correlated with fungal diversity, and fungal diversity had a positive regulatory effect on the plant antioxidant system. Under moderate drought (MD) conditions, the positive driving effect of soil pH on soil enzyme activity weakened, but it became negatively correlated with bacterial diversity, and bacterial diversity still significantly enhanced plant nutrients while

significantly reducing the accumulation of osmotic regulatory substances. Plant nutrients were significantly positively correlated with fungal diversity, the plant antioxidant system had a strong negative inhibitory effect on plant biomass, and the accumulation of antioxidant substances significantly inhibited plant growth. Under severe drought (SD) conditions, the positive effect of regulation of soil pH on soil enzyme activity further weakened; the negative correlation between pH and bacterial diversity weakened; bacterial diversity significantly enhanced the positive induction effect of osmotic regulatory substances; osmotic regulatory substances significantly promoted plant biomass, becoming the core pathway for maintaining plant survival under severe drought; and the accumulation of antioxidant substances promoted plant growth.

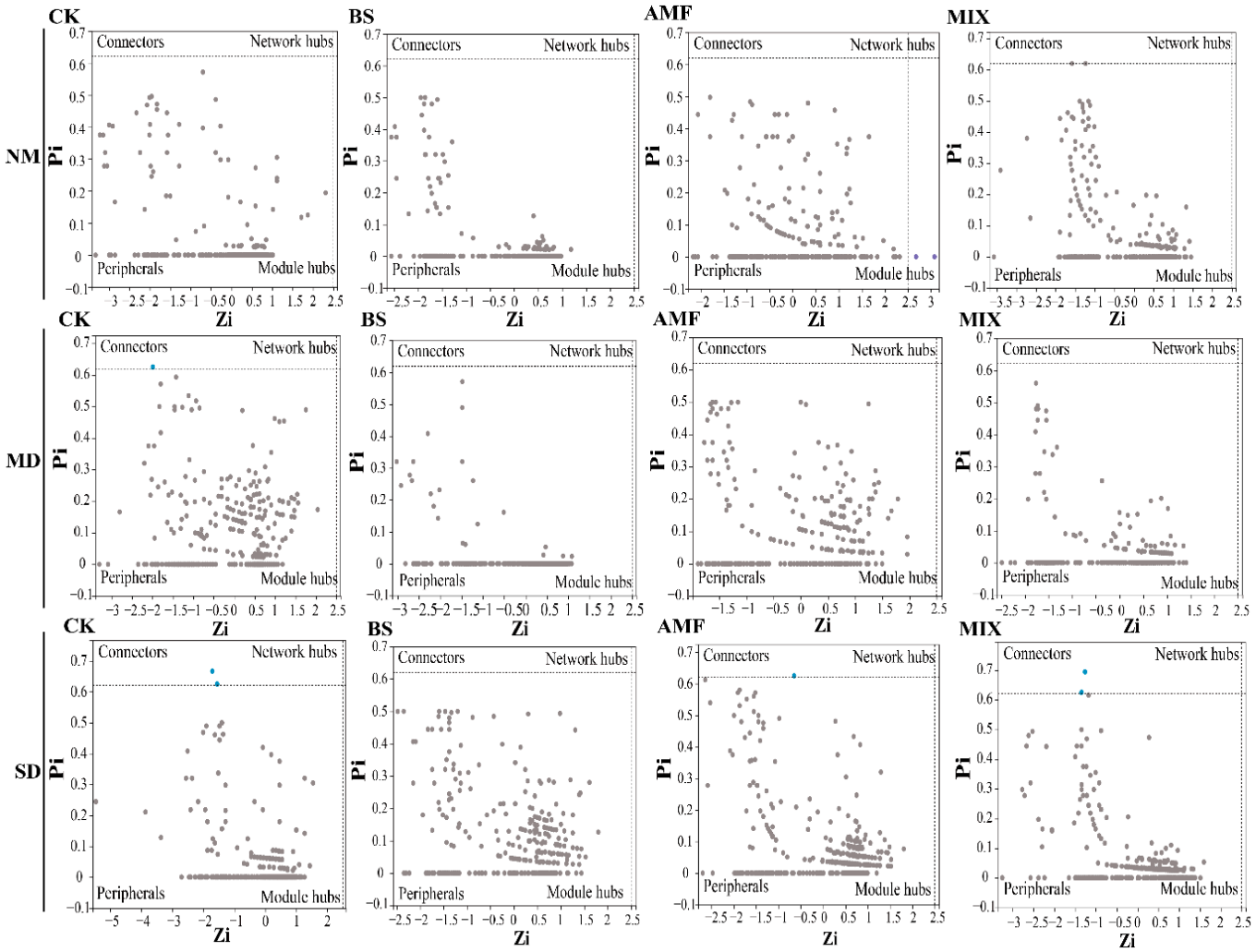


Figure 8. Within-module connectivity (*Zi*) and among-module connectivity (*Pi*) of soil fungal networks. Note: Nodes represent taxa or functions and are classified by topology into four categories: module hubs ($Zi > 2.5$ and $Pi < 0.65$), connectors ($Zi < 2.5$ and $Pi > 0.65$), network hubs ($Zi > 2.5$ and $Pi > 0.65$), and peripherals ($Zi < 2.5$ and $Pi < 0.65$). The dashed lines indicate the thresholds of Zi = 2.5 and Pi = 0.65. All categories except peripherals are considered keystone nodes. CK, sterilized preparations of both inoculants; BS, live *B. subtilis* plus sterilized AMF inoculum; AMF, live AMF plus sterilized *B. subtilis* preparation and MIX live *B. subtilis* plus live AMF. NM, Normal Moisture; MD, Moderate Drought; SD, Severe Drought.

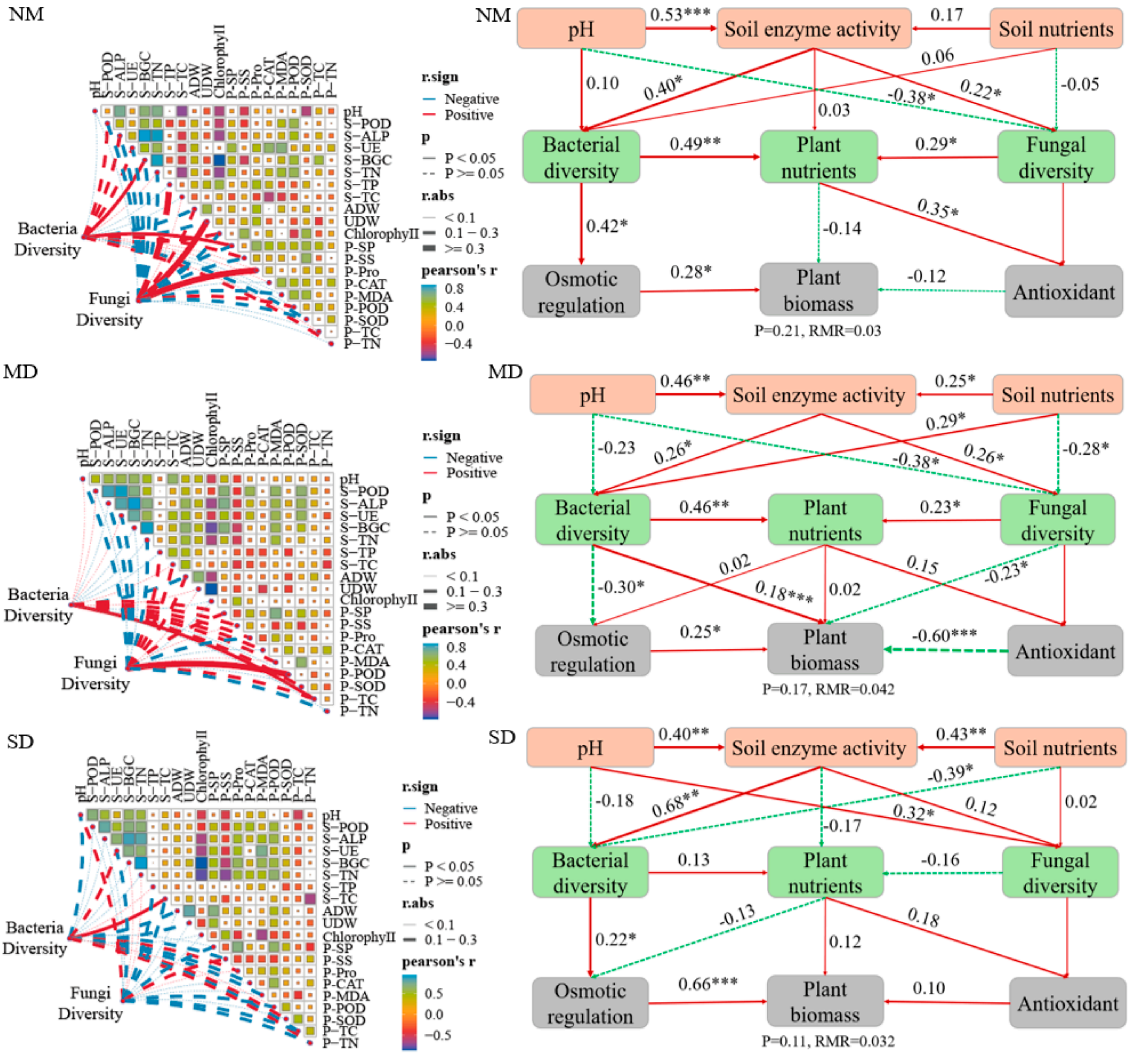


Figure 9. Soil–microbe–plant interactions. Note: Solid red arrows indicate significant positive paths, dashed green arrows indicate negative paths, and numbers beside arrows are standardized path coefficients. Significance is denoted by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. P-Pro, plant proline; P-SP, plant soluble protein; P-SS, plant soluble sugar; P-MDA, plant malondialdehyde; P-SOD, plant superoxide dismutase; P-POD, plant peroxidase; P-CAT, plant catalase; P-TC, plant total carbon; P-TN, plant total nitrogen; S-TC, soil total carbon; S-TN, soil total nitrogen; S-TP, soil total phosphorus; S-UE, soil urease; S-ALP, soil alkaline phosphatase; S-β-GC, β-glucosidase; S-POD, soil peroxidase; ADW, aboveground dry weight; UDW, belowground dry weight. NM, Normal Moisture; MD, Moderate Drought; SD, Severe Drought.

4. Discussion

4.1. Effects of Microbial Inoculation on Physiological Traits of Achnatherum splendens Under Drought Stress

This section primarily addresses our first core research question: whether single and combined inoculation with *Bacillus subtilis* (BS) and *arbuscular mycorrhizal fungi* (AMF) can improve the physiological performance of *A. splendens* under varying drought intensities.

Drought impairs plant growth by disrupting cellular water relations, limiting nutrient acquisition and transport, and suppressing photosynthetic carbon assimilation [21]. Consistent with these physiological mechanisms, both above- and belowground biomass of *A. splendens* declined progressively with increasing drought severity. Reduced soil water potential elevates hydraulic resistance to root water uptake and diminishes cellular turgor pressure—key drivers of cell division and elongation. Concurrently, diminished

diffusion and mass flow of nitrogen, phosphorus, and other essential nutrients toward roots constrain root-system development and generate negative feedbacks that further suppress shoot growth [22,23]. *Bacillus subtilis* (BS) and *Arbuscular mycorrhizal* fungi (AMF) are well-established plant growth-promoting microorganisms that enhance host tolerance to abiotic stressors including drought [24]. In line with our hypothesis, under normal moisture (NM), BS and MIX significantly increased above-ground biomass relative to CK ($p < 0.05$), with MIX showing the strongest enhancement. However, no significant increases in above-ground biomass were detected for any inoculation treatment under moderate drought (MD) or severe drought (SD). A mechanistic interpretation consistent with these observations is reciprocal facilitation: AMF hyphae may create protected microhabitats that enhance bacterial colonization and persistence in the rhizosphere, while *B. subtilis* may modulate root exudation patterns, solubilize mineral nutrients, and alter local redox conditions, thereby improving the biophysical environment for AMF establishment. This synergistic model aligns with prior evidence from dual-inoculation studies [25,26]; however, direct quantification of AMF colonization rates, bacterial root adherence, and cross-kingdom metabolite exchange was not performed and remains a critical target for future validation.

Osmotic adjustment and antioxidant defense are complementary components of plant drought response. Proline, soluble sugars, and soluble proteins contribute to cellular water balance and membrane stability [27,28], while SOD, POD, and CAT remove reactive oxygen species [29]. In this study, osmolyte concentrations and antioxidant enzyme activities exhibited variable responses to drought and inoculation across indicators and moisture regimes. Some osmolytes and antioxidant enzymes were elevated under drought and further modified by inoculation, with MIX showing pronounced effects for certain indicators. However, MIX did not consistently maintain the highest proline or enzyme activities; soluble sugars in MIX were lower than CK, and enzyme activities did not increase progressively with drought severity. These context-dependent physiological responses may partially explain the biomass differences observed in inoculated plants [30,31]. The MDA pattern, however, requires caution: inoculated plants had higher MDA than CK within moisture regimes, which is not straightforward evidence of reduced lipid peroxidation. Instead, it may result from symbiosis-triggered lipid remodeling and controlled lipid peroxidation, which can act as signaling events during plant–microbe interactions. The robust antioxidant system (SOD, POD, CAT, and osmoprotectants) can effectively scavenge excess ROS and prevent the accumulation of toxic oxidative products, thereby preserving plant biomass under drought. Similar uncoupling between MDA level and oxidative injury has been reported in previous AMF and PGPR inoculation studies. Overall, the physiological data support improved osmotic and antioxidant capacity following inoculation, but direct claims about gene regulation or membrane protection require additional measurements [32]. Notably, this study suggests a possible antagonistic effect of dual MIX on chlorophyll synthesis, which contrasts sharply with the synergistic growth-promoting and stress-alleviating effects of MIX observed in plant biomass, osmotic adjustment, and antioxidant defense. Specifically, combined inoculation with *B. subtilis* and AMF was observed to significantly reduce leaf chlorophyll content compared to single inoculants and the non-inoculated control across all soil moisture regimes. This observation raises a hypothesis of a context-specific negative interaction between the two microbial strains that may affect plant photosynthetic pigment metabolism.

Importantly, this seemingly contradictory result—lower chlorophyll content accompanied by improved plant biomass—requires reasonable mechanistic interpretation, as reduced chlorophyll typically causes leaf chlorosis and growth inhibition under stress. We propose two mutually compatible hypothetical explanations for this paradox. First, it

is hypothesized that MIX-induced chlorophyll reduction is a regulated photoprotective acclimation strategy rather than irreversible physiological damage. Moderately reduced chlorophyll content alleviates excessive light absorption under drought stress, mitigating photoinhibition and ROS burst, while the enhanced antioxidant system offsets the potential photosynthetic disadvantages. Second, the dual inoculation may substantially improve root water and nutrient acquisition capacity and strengthens osmotic adjustment ability. These comprehensive stress-resistant advantages could potentially compensate for the partial decline in photosynthetic pigment content, thereby sustaining and promoting plant biomass accumulation. We hypothesize that this physiological trade-off between chlorophyll synthesis and drought adaptation represents a stress acclimation strategy of *A. splendens* responding to combined microbial inoculation and drought stress, rather than pathological deterioration.

4.2. Effects of Microbial Inoculation on Soil Nutrients and Enzyme Activities Under Drought Stress

This section addresses our third research question, focusing on how drought intensity modulates the soil–microbe–plant functional pathways related to plant biomass regulation under single and combined microbial inoculation. Soil nutrient pools and extracellular enzyme activities provide complementary yet mechanistically distinct insights into rhizosphere biogeochemical functioning [33]. Total carbon (S-TC), nitrogen (S-TN), and phosphorus (S-TP) concentrations showed no significant variation across the drought severity gradient, consistent with the inherent stability and slow turnover rates characteristic of bulk soil nutrient pools.

Inoculation exerted variable effects on S-TC, S-TN, and S-TP relative to CK; some inoculation treatments increased these nutrient pools under certain moisture regimes, whereas neutral or even negative responses were observed in other treatment combinations. Inoculation exerted variable effects on S-TC, S-TN, and S-TP relative to CK; some inoculation treatments increased these nutrient pools under certain moisture regimes, whereas neutral or even negative responses were observed in other treatment combinations. These context-dependent shifts in soil nutrients may imply potential changes in microbial biomass, organic matter stabilization, or nutrient leaching; however, these underlying processes were not directly measured in this study [34,35]. In contrast, urease (S-UE) and β-glucosidase (S-β-GC) were significantly affected by drought severity, while alkaline phosphatase (S-ALP) and peroxidase (S-POD) showed no significant response to drought. Inoculation generally increased the activities of S-UE, S-ALP, S-β-GC and S-POD; MIX produced the strongest enhancement relative to CK for most of these enzymes ($p < 0.05$). Water deficit induces microbial reallocation of resources toward extracellular enzyme synthesis to compensate for impaired substrate diffusion and reduced pore connectivity, whereas microbial inoculants can modulate both the taxonomic composition and functional expression of rhizosphere communities [36–38]. Consequently, the observed enzyme responses are consistent with microbially mediated adjustments in nutrient acquisition pathways but do not provide direct evidence of altered net mineralization, immobilization, or turnover rates.

Drought and inoculation exhibited significant main effects on S-UE activity, but their interaction was not significant. For S-β-GC, both main effects and their drought × inoculation interaction were statistically significant ($p < 0.05$; Table 3). As S-UE catalyzes the hydrolysis of urea to ammonium, a rate-limiting step in organic nitrogen mineralization, and S-β-GC mediates the cleavage of β-glycosidic bonds in cellulose-derived oligosaccharides, a key bottleneck in soil carbon depolymerization, these enzymes serve as sensitive functional indicators of N and C bioavailability. Their heightened responsiveness to drought and inoculation, relative to S-ALP and S-POD, likely reflects greater sensitivity to shifts in water potential, substrate diffusion limitations, and the abundance

of specific enzyme-producing taxa (e.g., Bacillus, Pseudomonas, and saprotrophic fungi). The significant drought × inoculation interaction for S-β-GC further demonstrates that the magnitude of inoculant-induced enzyme stimulation is contingent upon prevailing moisture conditions [39]. Collectively, the congruent shifts in plant physiological traits (e.g., osmotic adjustment and antioxidant activity), soil enzyme activities, and microbial co-occurrence network structure may indicate system-wide reorganization of the rhizosphere under drought stress; however, longer-term experiments are required to determine whether these short-term functional adjustments translate into sustained increases in plant-available nutrient supply and improved agronomic performance [40].

4.3. Effects of Microbial Inoculation on Soil Microbial Communities Under Drought Stress

This section systematically answers our second core research question: whether soil bacterial and fungal diversity and community structure exhibit consistent responses to BS, AMF, and MIX across different drought intensities. Bacterial richness (Chao1) was significantly higher under MD compared with NM, while SD did not differ significantly from either NM or MD. The bacterial Shannon index was significantly lower under NM than under MD and SD ($p < 0.05$). Water deficit can alter soil pore connectivity and restrict nutrient diffusion; such environmental filtering may select for drought-tolerant, oligotrophic bacterial taxa [41]. Nonetheless, elevated diversity under stress cannot be directly interpreted as enhanced ecosystem functioning because community responses may be governed by functional redundancy or compensatory shifts among rare taxa. Inoculation tended to increase bacterial Chao1 and Shannon indices relative to CK, though significant differences were only detected for selected inoculation treatments within individual moisture regimes. Mechanisms potentially underlying these diversity shifts may include expansion of access to spatially isolated soil microsites and immobile nutrients via AMF hyphae [10], or cross-kingdom synergies such as bacterial modulation of mycorrhizal signaling or AMF promotion of bacterial persistence in the rhizosphere [42]. However, these proposed interactions remain speculative in the present study. Fungal richness and alpha diversity were comparatively stable across moisture regimes. This pattern is consistent with the capacity of fungal hyphae to traverse air-filled pores in dry soil and the structural resilience of chitin-rich fungal cell walls [43]. However, such physiological traits are not directly quantified in our experiment. Principal coordinates analysis (PCoA) of Bray–Curtis dissimilarities confirmed that both drought severity and microbial inoculation drove significant shifts in overall bacterial and fungal community composition ($p < 0.01$). These findings support water availability as a primary environmental filter shaping microbial assembly; however, taxon-specific response patterns and their functional implications remain unresolved and warrant targeted metatranscriptomic or stable-isotope probing approaches [44,45].

Network analysis revealed a systematic trade-off between network connectivity (edge count) and the proportion of positive correlations. MIX consistently generated the largest bacterial co-occurrence networks across all moisture regimes, potentially reflecting inoculant-driven expansion of rhizosphere resource heterogeneity and modulation of chemical signaling (e.g., quorum-sensing molecules and strigolactones) [46]. However, MIX did not maximize the proportion of positive bacterial edges: CK exhibited the highest proportion under NM (59.7%), BS under MD (60.8%), and AMF under SD (55.4%). Fungal networks displayed divergent moisture-dependent structuring: AMF yielded the largest and most positively correlated network under NM, CK produced the largest network under MD, and BS generated the largest network under SD. Collectively, these regime-specific configurations align with the theory of context-dependent microbial assembly, wherein deterministic selection pressures shift in strength and identity across environmental gradients [47–49]. Critically, co-occurrence edges are derived from pairwise

correlation coefficients (e.g., SparCC and Spearman) and represent statistical associations, not empirically observed ecological interactions. Consequently, network size and edge sign must be interpreted as testable hypotheses regarding potential cooperative or competitive organization, not as direct evidence of biological interaction. These insights motivate field-based evaluation of moisture-matched inoculation strategies (e.g., AMF under well-watered conditions and BS under drought) rather than prescriptive recommendations for universal application.

Zi–Pi topological analysis further clarified the structural traits of microbial networks. Bacterial networks across all treatments were dominated by peripheral nodes, with a small number of connector taxa and rare module hubs, and no network hubs were detected. Inoculation effectively increased the number of connectors and mean *Pi* values, and MIX treatment possessed more key nodal taxa than single inoculation, indicating that combined inoculation optimizes the modular connectivity of bacterial networks under drought. For fungal networks, peripheral nodes overwhelmingly dominated all treatments, with extremely limited inter-module connectivity and no core network hubs. This simple and loose topological pattern suggests a potential constraint on inter-module associations among fungal taxa under drought stress.

Collectively, the findings of this study fully verify that the growth-promoting and stress-alleviating effects of BS and AMF inoculation are highly dependent on drought intensity. The plant physiological adaptation, soil nutrient cycling, and microbial community assembly regulated by single and combined inoculation show distinct moisture-specific response patterns. These results provide a targeted theoretical basis for precise microbial inoculation strategies in dryland ecological restoration and indicate that universal inoculation schemes are not applicable, while moisture-matched inoculation modes (AMF for well-watered conditions and BS for severe drought) have greater application potential.

This study employed a single plant genotype (*A. splendens*), standardized growth substrate (sterilized humus soil), and a four-week greenhouse drought treatment. Although these controlled conditions enabled precise inference of the mechanism, they inherently limited the extrapolation of the results to heterogeneous soils and multi-seasonal ecological restoration effects. Amplicon sequencing can provide reliable taxonomic resolution, but it cannot directly quantify microbial metabolic activity, gene expression, or the frequency of physical interactions. Moreover, although in controlled pot experiments, single-variable statistical comparisons (such as analysis of variance ANOVA) typically use four biological replicate samples, multivariate modeling methods like PLS-PM and microbial network inference require larger datasets to achieve stable parameter estimation and reliable association inference.

5. Conclusions

This study shows that drought stress significantly inhibits biomass accumulation of *Achnatherum splendens*, alters plant physiological metabolism and rhizosphere microecology, and remodels the multi-level soil–microbe–plant regulatory pathway. Inoculation with *Bacillus subtilis* (BS) and arbuscular mycorrhizal fungi (AMF) improves antioxidant and osmotic adjustment capacities of *A. splendens* and enhances rhizosphere soil nutrients and soil enzyme activities to mitigate drought damage, with mixed inoculation (MIX) exhibiting the optimal synergistic effect. Drought and inoculation jointly shape rhizosphere microbial communities and co-occurrence networks; bacteria are more sensitive to drought. Microbial adaptability varies across water gradients, and osmotic adjustment serves as the core survival strategy for *A. splendens* under severe drought. This work provides theoretical support for ecological restoration using *A. splendens*. However, conclusions are inferred from indirect sequencing and enzyme measurements, without quantifying

AMF root colonization and in vivo bacterial dynamics, so the underlying mechanisms need further verification.

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