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Water availability modulates how *Bacillus subtilis* promotes alfalfa growth under salinity stress: coordinated physiological and rhizosphere responses

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Abstract

Background

Soil water availability can influence plant responses to salinity and may determine the effectiveness of plant growth-promoting rhizobacteria (PGPR). However, how water availability modulates PGPR-mediated plant and rhizosphere responses under salinity stress remains unclear. A pot experiment was conducted to evaluate alfalfa (Medicago sativa L.) responses to *Bacillus subtilis* inoculation under two salinity levels (3.0 and 4.5 g kg^{-1}) and two soil moisture regimes (55-70% and 70-85% of field capacity).

Results

B. subtilis increased alfalfa dry biomass by 10.31-188.43% relative to the corresponding non-inoculated treatments. Inoculation increased catalase activity and reduced proline and malondialdehyde accumulation, indicating alleviation of physiological stress. These responses were accompanied by lower rhizosphere electrical conductivity and NH_4^+-N concentrations and generally higher soil organic matter, total nitrogen, and NO_3^--N availability. Activities of urease, glutamine synthetase, nitrate reductase, and nitrogenase also increased following inoculation. Bacterial α-diversity did not differ significantly among treatments, whereas community structure varied with water and salinity conditions. Redundancy analysis identified nitrate reductase activity, total nitrogen, electrical conductivity, NO_3^--N, and NH_4^+-N as important variables associated with bacterial community structure. Exploratory PLS-SEM further indicated that reduced salinity and

improved rhizosphere nutrient conditions were closely associated with enhanced alfalfa growth. The most favorable overall response occurred at 3.0 g kg^{-1} salinity and 70-85% field capacity.

Conclusions

B. subtilis improved alfalfa performance under combined water and salinity stress, but its effectiveness depended on soil water availability and salinity severity. Coordinating PGPR application with appropriate water management may therefore improve alfalfa production in salt-affected environments.

Key words: *Bacillus subtilis*; Water and salt stress; Soil physical and chemical properties；Soil enzyme activity

1 Introduction

Global agriculture's sustainable growth is seriously hampered by the deterioration of arable land quality brought on by soil salinization and alkalization. According to a recent worldwide assessment report published by the Food and Agriculture Organization of the United Nations, the area of land impacted by salt stress has reached 13.81 billion hectares, or 10.7% of the total land area [1]. Excessive soil salinity frequently results in ionic and osmotic stress in plants [2, 3], leading to oxidative stress, nutritional problems, and organ senescence in plants, which in turn causes irreversible and significant losses, with yield reductions of up to 80% [2-4]. Therefore, while ensuring sufficient arable land and food security, preventing soil degradation and enhancing crop resilience are key measures for maintaining sustainable agricultural development. Changing farming practices—such as crop rotation, fallowing, and converting cropland to grassland are typically effective measures for restoring soil fertility and preventing soil degradation [5-7]. Alfalfa is a moderately salt-tolerant legume that can improve soil fertility and crop nutrition through biological nitrogen fixation. Through symbiotic nitrogen fixation, alfalfa can obtain approximately 65% of the nitrogen required for its growth, thereby substantially reducing the need for inorganic nitrogen fertilizers [8]. Most grower plant alfalfa on sandy soils or saline-alkali land with low fertility to improve soil fertility [5, 7, 8]. This measure has been implemented in China for more than 20 years [9], during which large areas of poor-quality farmland, including saline–alkali land, have been converted into forage fields or pastures [10, 11]. Alfalfa is widely grown in

northwestern China, however the sustainable development of agricultural and animal husbandry is negatively impacted by water scarcity and soil salinization.

It is well known that plant responses to water and salt stress involve complex physiological and biochemical changes and plants can enhance their tolerance by modifying their morphology and regulating physiological and biochemical responses [5, 12-14]. Moderate water deficit can trigger adaptive regulatory responses in crops, leading plants to prioritize biomass allocation to roots, thereby increasing the root-to-shoot ratio and enhancing water uptake and plant performance [13, 14]. Salt stress is very similar to water stress; under moderate salt stress (2-6 $dS \cdot m^{-1}$), alfalfa can increase fresh forage yield in the short term by enhancing antioxidant enzyme activity, accumulating osmoregulatory substances, and maintaining K^+ homeostasis in leaves. However, under high salinity stress(>6-10 $dS \cdot m^{-1}$), alfalfa growth is markedly inhibited, with increased Na^+ accumulation, impaired K^+ uptake, reduced photosynthetic efficiency and chlorophyll content, and disrupted respiratory metabolism, ultimately leading to substantial productivity losses [5]. In addition to their intrinsic systems for protecting themselves from environmental stress, research has shown that plants can also form beneficial associations with certain microorganisms in the rhizosphere, thereby mitigating the damage caused by environmental stress.

In recent years, as research on soil decomposers, particularly microorganisms, has deepened, studies have shown that rhizosphere microorganisms can help host plants withstand abiotic stress by regulating physiological and biochemical processes such as intracellular ion balance, osmotic adjustment, and the levels of plant hormones and antioxidant compounds [15, 16]. This offers new approaches to addressing challenges such as soil salinization and water scarcity. Plant growth-promoting rhizobacteria (PGPR) are a group of beneficial rhizosphere microorganisms that play a crucial role in enhancing crop tolerance to abiotic stresses, including drought, salinity, and heavy metal stress [17, 18]. PGPR not only directly promote plant growth by synthesizing and secreting phytohormones, but also enhance plant tolerance by regulating endogenous hormone levels [19]. Under drought stress, PGPR can form hydrophilic biofilms around plant roots by producing extracellular polysaccharides (EPS), thereby enhancing root water retention [20], alternatively, PGPR can activate plant antioxidant defense mechanisms by inducing the synthesis of superoxide dismutase (SOD) and peroxidase (POD), thereby reducing reactive oxygen species (ROS) induced damage in leaves and significantly increasing crop germination rates under drought stress [21].

Lee et al. [22] showed that some salt-tolerant PGPR, such as *B. cereus*, *Promicromonospora* sp., and *Enterococcus faecium*, can secrete gibberellic acid (GA). After inoculation, these strains can increase GA levels in plant roots and alleviate salt stress-induced damage. Moreover, PGPR strains can enhance soil nutrient availability and regulate the rhizosphere soil environment. For instance, *Azospirillum brasilense* and *Pseudomonas fluorescens* have been reported to promote soil nitrogen mineralization under salt stress, enhance nitrogenase activity, accelerate nitrogen transformation, and improve the nitrogen-supplying capacity of rhizosphere soil, thereby significantly increasing rice biomass [23]. Soil inoculation of cherry tomato with *Bacillus cereus* DW019 modulates the diversity and structure of the rhizosphere microbial community, particularly the relative proportions of *Proteobacteria* and *Actinobacteria*, thereby improving plant height, fresh weight, fruit nutritional quality, and bacterial diversity in the rhizosphere soil [24]. This multi-strain blend releases organic acids, which significantly increase nutrient levels and enzyme activity in the soil, thereby influencing the rhizosphere microbial community and providing better nutrition to promote plant growth [25, 26]. Previous studies have primarily focused on the regulatory effects of PGPR on plant physiological and biochemical responses under water or salt stress alone. However, research on how PGPR enhances plant tolerance and improves soil health through cross-adaptation mechanisms under combined water and salt stress remains limited.

B. subtilis is a PGPR that is widely present in the natural environment. It can improve the rhizosphere environment, promote the absorption of water and nutrients by crops, and increase crop yields. Therefore, its application in agricultural production is becoming increasingly widespread [27-29]. Given its importance, this study selected *B. subtilis* for pot experiments, aiming to explore its effects on the growth and physiological characteristics of alfalfa, soil physical and chemical properties, rhizosphere enzyme activity, and bacterial communities under combined water and salt stress conditions. To reveal the mechanism by which *B. subtilis* enhances the stress resistance of alfalfa and regulates the rhizosphere soil environment. At the same time, to comprehensively evaluate the effects of *B. subtilis* on the physiology and growth of alfalfa and the improvement of soil health. This research aims to provide theoretical basis and technical support for improving the productivity of saline-alkali land, saving water resources, and promoting sustainable agricultural development.

2 Materials and Methods

2.1 Experimental design

The pot experiment was conducted in 2024 at Inner Mongolia University of Science and Technology, China (40°37'4.8"N， 109°49'44.4"E). Seeds of the alfalfa cultivar 'Algonquin' (Medicago sativa L.) were purchased from Yanjie Seed Business Department, Helan County, Ningxia Hui Autonomous Region, China, which has strong cold and drought tolerance and excellent regrowth capacity. Soil samples were collected from an experimental field in Wuyuan County, Inner Mongolia, China (41°5′N，108°16′E). The soil electrical conductivity (EC) was 0.57 $dS \cdot m^{-1}$, the pH was 7.89, the total nitrogen (TN) was 0.98 $g \cdot kg^{-1}$, the total phosphorus content was 0.55 $g \cdot kg^{-1}$, and the soil organic carbon (SOC) was 2.35 $g \cdot kg^{-1}$. The soil was air-dried in the shade for one week, sieved through a 4 mm mesh, thoroughly mixed, and stored at 4°C until use. Seedlings were raised in plug trays containing the same soil as that used in the pot experiment. After four weeks, seedlings with 3–4 leaves were transplanted into plastic pots measuring 24.5 cm in diameter and 30 cm in height. Each pot was filled with 6 kg of sieved soil, and a plastic tray was placed beneath each pot to prevent water and fertilizer loss. Twenty seedlings were planted in each pot, and each pot was wrapped with 20 mm thick insulating cotton to reduce the influence of external temperature on soil water evaporation. Three replicate pots were established for each treatment, and all pots were arranged in a completely randomized design, giving a total of 27 pots. Moreover, compound fertilizer with an N: P: K ratio of 1:1:1 was applied at a rate equivalent to 0.75 t ha^{-1} and thoroughly mixed with the soil using a shovel. During the alfalfa growing period, irrigation was applied through a polyethylene hose with a diameter of 1 cm. The polyethylene hose was equipped with a diaphragm pressure compensating emitter with a labyrinth flow path. The operating pressure was 0.05-0.20 MPa, and the flow rate was 1.0 $L \cdot h^{-1}$. This system reduced water pressure and prevented soil and substrate scouring during irrigation. The same management practices were applied to all treatments.

This experiment used a three-factor design, with salinity (S), soil moisture (W), and *B. subtilis* application (P) as treatment factors. Two soil moisture levels were established: W1, 55–70% of field capacity, and W2, 70–85% of field capacity. Based on the actual situation of local soil type, mixed salts of NaCl and Na_2SO_4 were prepared with the same weight for the experiment (NaCl :

Na_2SO_4 = 1:1 [w/w]). The salt addition rates were 0.3% and 0.45% of soil dry weight, corresponding to 3.0 and 4.5 $g \cdot kg^{-1}$ dry soil, and were referred to as S1 and S2, respectively. Before salt addition, the mixed salts were dissolved in water according to the designed salinity levels and then uniformly applied to the soil. Each water and salt stress treatment was supplemented with or without *B. subtilis*. The bacterial agent employed was *B. subtilis* in form of wettable powder, containing 100 billion $CFU \cdot g^{-1}$. The first root drench was applied 2 days after the onset of salt stress. For each pot, 0.2 g of the microbial agent was diluted in 100 mL of distilled water and evenly applied to the root zone. For the corresponding treatments without B. subtilis application, the same volume of distilled water was applied at each application time. Subsequent root drenches were applied every 20 days, for a total of three applications, resulting in a cumulative application rate of 0.6 g per pot. A separate control treatment (CK) was established without salt addition, and soil moisture was maintained at 85–100% of field capacity. Thus, a total of nine treatments were established, with three replicate pots per treatment. During the water control period, pot weight was measured daily using an electronic balance with an accuracy of 0.01 g, and tap water was added at 8:00 a.m. to maintain the variation in relative soil moisture content within 5%. At the early flowering stage, ten plants with uniform growth were selected from each pot to determine plant height, aboveground biomass, and antioxidant enzyme activities.

Table 1. Experimental design details for different treatments.

Treatment	Water treatment	Salt treatment ($g \cdot kg^{-1}$)	Bacillus subtilis application rate ($g \cdot pot^{-1}$)
S1W1	55%-70%FC	3.0	0
S1W2	70%-85%FC	3.0	0
S2W1	55%-70%FC	4.5	0
S2W2	70%-85%FC	4.5	0
S1W1-P	55%-70%FC	3.0	0.6
S1W2-P	70%-85%FC	3.0	0.6
S2W1-P	55%-70%FC	4.5	0.6
S2W2-P	70%-85%FC	4.5	0.6
CK	85%-100%FC	0	0

Note: W1 and W2 represent soil moisture regimes of 55–70% and 70–85% of field capacity, respectively. S1 and S2 represent salt addition rates of 3.0 and 4.5 g kg^{-1} dry soil, respectively. The mixed salt consisted of NaCl and Na_2SO_4 at a mass ratio of 1:1. P indicates *B. subtilis* inoculation. The inoculant was applied at 0.2 g pot^{-1} per application for three applications, resulting in a cumulative dose of 0.6 g pot^{-1}. CK represents the well-watered control without added salt or *B. subtilis*.

2.2.1 Measurement of plant growth, physiological, and biochemical parameters

At the initial flowering stage, plant height was measured from the stem base to the shoot apex using a ruler. The aboveground parts were cut at a stubble height of 5 cm and weighed to determine fresh weight. A subsample of fresh material was immediately frozen in liquid nitrogen, transported to the laboratory, and stored at −80 °C for antioxidant enzyme assays. The remaining fresh material was placed in envelopes, transported to the laboratory, air-dried to constant weight, and weighed to determine aboveground dry weight.

Plant physiological traits were determined using assay kits (Zhiyi Biotechnology （Taizhou)Co., Ltd., China).The kits used were as follows: Superoxide Dismutase (SOD) Activity Assay Kit (WST-8 method, catalog number UPLC-MS-5048), Catalase (CAT) Activity Assay Kit (UV spectrophotometric method, catalog number UPLC-MS-4533), Malondialdehyde (MDA) Assay Kit (thiobarbituric acid method, catalog number UPLC-W-A401), and Proline Assay Kit (acidic indigo carmine method, catalog number UPLC-W-A605). All procedures were performed strictly in accordance with the kit instructions.

2.2.2 Soil Sampling

Soil samples in pots were collected 2 days before cutting. The soil samples were collected from the soil surface to the bottom of each pot using a 12 mm diameter auger. The samples were placed in a cooler with ice packs and transported to the laboratory for processing. Samples from each treatment were divided into three subsamples: one was air-dried at room temperature for the determination of soil physicochemical properties, one was stored at 4 °C for no more than one week for soil enzyme activity assays [30]. The remaining subsample was stored at −80 °C for subsequent microbial community analysis. After soil sampling, the sampling holes were immediately backfilled with the same soil used in the experiment.

2.2.3 Soil Physicochemical Properties and Enzyme Activities

After collection, soil samples were gently broken apart, air dried, and passed through a 2 mm sieve. Soil electrical conductivity (EC) was measured in a soil water extract prepared at a ratio of soil to water of 1:5 using a conductivity meter (DDSJ-308F, Zhongxi Agricultural Technology Co., Ltd., China) after shaking and centrifugation. Soil organic matter (SOM) was determined using the potassium dichromate oxidation with external heating method, and TN was measured using the Kjeldahl method. Soil ammonium nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N) were

extracted with potassium chloride and determined by spectrophotometry according to HJ 634 2012 using a UV visible spectrophotometer (L6s, Shanghai Yidian Scientific Instrument Co., Ltd., China).

The activities of soil urease, nitrate reductase (NR), glutamine synthetase (GS), and nitrogenase were determined using ELISA kits according to the manufacturer's instructions (Zhiyi Biotechnology, Taizhou, China). Briefly, soil extracts, standards, and blanks were added to microplates coated with antibodies, followed by incubation with detection antibody labeled with HRP. After washing and color development, absorbance was measured at 450 nm, and enzyme activities were calculated from standard curves.

2.2.4 Soil microbial communities

Genomic DNA was extracted using the CTAB method, and DNA quality and concentration were checked by 1% agarose gel electrophoresis. The V4–V5 hypervariable region of the bacterial 16S rRNA gene was amplified using the primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′). PCR products were purified, pooled in equal amounts, and used for library construction. After quality assessment, paired-end sequencing was performed on the Illumina HiSeq 2500 platform. The 16S rRNA amplicon sequencing was used to characterize the overall rhizosphere bacterial community. The persistence and abundance of the inoculated B. subtilis strain were not specifically quantified.

2.2.5 Comprehensive evaluation of alfalfa physiology and growth

The growth and physiological indices of alfalfa, including plant height, fresh weight, dry weight, SOD, CAT, MDA and proline were selected, and principal component analysis and membership function analysis were used to evaluate the growth and physiological performance of alfalfa. The comprehensive evaluation score was calculated according to the following equations [31]:

$$U_n = \frac{X_n - X_{min}}{X_{max} - X_{min\ 0}} \quad (1)$$

$$V_n = \frac{P_n}{\sum_{n=1}^{n} P_n} \quad (2)$$

$$GPI = \sum_{n=1}^{n} (U_n \times V_n) \quad (3)$$

Where X_n is the nth comprehensive index value of each treatment; X_{min} and X_{max} are the minimum and maximum values of the nth comprehensive index among all treatments, respectively;

234 U_n is the membership function value of the nth comprehensive index of each treatment; P_n is the
235 eigenvalue of the nth principal component; V_n is the weight of the nth principal component; and
236 GPI is the comprehensive evaluation score of alfalfa growth and physiology.

237 2.2.6 Quantification of soil quality and ecosystem multifunctionality

238 The selected soil physicochemical indicators included EC, TN, SOM, NH_4^+-N and NO_3^--N;
239 the soil enzyme indicators included urease, NR, GS and nitrogenase; and the microbial diversity
240 indicators included Chao1 and Shannon. The total data set (TDS) method was used to calculate
241 SQI [32].First, all indicators were standardized to a range of 0 to 1. The indicators were classified
242 into two categories according to their relationships with soil quality. Indicators positively
243 associated with soil quality, such as TN and SOM, were standardized according to Eq. (4), whereas
244 indicators negatively associated with soil quality, such as EC, were standardized according to Eq.
245 (5) [33, 34].Subsequently, the communality of each indicator was extracted by principal
246 component analysis, and the weight (E_j) of each indicator was calculated as the ratio of its
247 communality to the sum of the communalities of all indicators. After all indicators were scored
248 and weighted, SQI was calculated according to Eq. (6).

249 $$Q_i = \frac{Z - Z_{min}}{Z_{max} - Z_{min}} \quad (4)$$

250 $$Q_j = \frac{Z_{max} - Z}{Z_{max} - Z_{min}} \quad (5)$$

251 $$SQI = \sum_j^n (Q_j \times E_j) \quad (6)$$

252 Where Q_j is the standardized score of the jth soil indicator; Z_j is the measured value of the
253 jth soil indicator; Z_{max} and Z_{min} are the maximum and minimum values of the jth soil indicator
254 among all treatments, respectively; E_j is the weight of the jth soil indicator; and n is the total
255 number of indicators.

256 2.2.7 Plant-soil system health index (PSI)

257 To comprehensively evaluate the overall effects of different treatments on alfalfa growth and
258 physiology and soil quality, GPI and SQI were assigned equal weights. The plant–soil system
259 health index (PSI) was calculated according to the following equation：

260 $$PSI_i = 0.5 \times GPI_i + 0.5 \times SQI_i \quad (7)$$

2.3 Statistical analysis

The experimental data were processed and analyzed using IBM SPSS Statistics 24.0 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to compare treatment means at $P < 0.05$. Three-way ANOVA was performed to evaluate the main and interactive effects of water treatment (W), salinity treatment (S), and *B. subtilis* treatment (P) on the measured parameters. Pearson correlation analysis was conducted to examine the relationships among alfalfa growth and physiological traits, soil nutrients, and soil enzyme activities. Detrended correspondence analysis (DCA) was first conducted using Canoco 5.0 (Microcomputer Power, Ithaca, NY, USA), followed by redundancy analysis (RDA, linear model) to evaluate the relationships between soil bacterial community composition and environmental variables. Partial least squares structural equation modeling (PLS-SEM) was used as an exploratory analysis to examine statistical associations among PGPR application, soil salinity, soil nutrient status, soil enzyme activities, bacterial diversity, and alfalfa growth. Figures were generated using Origin 2024 (Origin Lab Corporation, Northampton, MA, USA).

3 Results

3.1 Effects of *Bacillus subtilis* application on alfalfa growth and physiology under water and salt stress

3.1.1 Alfalfa growth and dry weight

As a whole, the fresh weight and dry weight of alfalfa decreased significantly under combined water and salt stress ($P < 0.05$), but the impact on plant height was relatively minor (Fig. 1 a, b). The average plant height, fresh weight, and dry weight of alfalfa decreased by 7.58%, 35.85%, and 47.42% respectively. Under combined water and salt stress, treatments with B. subtilis application increased the plant height and dry weight of alfalfa. The plant height and dry weight were increased by 10.63% - 30.25% and 10.31% - 188.43% compared with the treatment without *B. subtilis* application. Among them, treatments with B. subtilis application had the most significant promoting effect on the growth and dry weight of alfalfa under the S2W1 treatment.

3.1.2 Alfalfa physiological parameters

The activities of SOD and CAT in alfalfa both increased under combined water and salt stress (Fig. 1c, d). The addition of *B. subtilis* significantly affected the activities of SOD and CAT in

alfalfa plants ($P < 0.05$). Under the same water and salt stress conditions, the SOD activity in alfalfa with S1W1-P treatment increased by 45.45% compared to the S1W1 treatment. However, when the soil salinity reached 4.5 g kg^{-1}, the application of *B. subtilis* reduced the SOD activity in alfalfa, which was 19.08% - 24.79% lower than the treatment without adding Bacillus subtilis. The addition of *B. subtilis* increased the CAT activity, which was 10.58% -27.61% higher than the treatment without *B. subtilis*.

Under combined water and salinity stress, proline and MDA contents in alfalfa showed similar response patterns in treatments with and without *B. subtilis* application, increasing with increasing salinity and decreasing soil water availability (Fig. 1e, f). Overall, the changes in proline and MDA were more pronounced with increasing salinity than with decreasing soil water availability. Compared with CK, the average proline contents under S2 and S1 increased by 289.41% and 128.54%, respectively, whereas the average MDA contents increased by 105.33% and 46.43%, respectively. Compared with the corresponding treatments without *B. subtilis* application, the treatments with *B. subtilis* application showed significantly lower proline and MDA contents, with reductions of 14.26-29.75% and 3.45-27.13%, respectively. The largest relative reductions in proline and MDA were observed under the S2W1 condition. These results indicate that *B. subtilis* application was associated with lower proline and MDA accumulation under combined water and salinity stress, consistent with reduced osmotic stress and membrane lipid peroxidation.

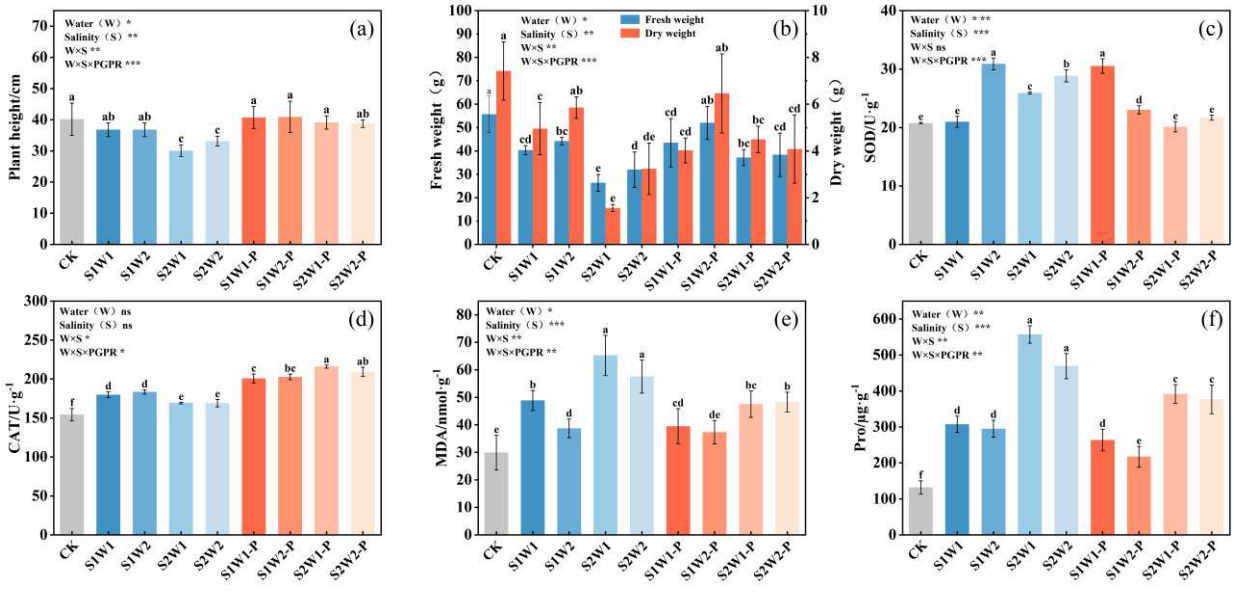


Fig. 1 Changes in growth and physiological indicators of alfalfa under different treatments (a plant height; b fresh weight and dry matter weight; c superoxide dismutase activity; d catalase activity; e

proline content; f malondialdehyde content). Different lowercase letters above bars indicate significant differences among treatments, $p < 0.05$. ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.2 Effects of Bacillus subtilis application on soil properties and enzyme activities under water and salt stress

3.2.1 Soil Physicochemical Properties

Fig. 2 shows the changes in rhizosphere soil physicochemical properties under different treatments. Overall, combined water and salinity stress increased rhizosphere soil EC and altered soil nitrogen status. Compared with CK, the average soil EC and NH_4^+-N content increased by 61.04% and 164.67%, respectively, whereas SOM, TN, and NO_3^--N contents decreased by 38.68%, 36.41%, and 45.20%, respectively. Under the same water and salinity conditions, the treatments with B. subtilis application generally showed lower rhizosphere soil EC and NH_4^+-N content and higher SOM, TN, and NO_3^--N contents than the corresponding treatments without B. subtilis application. A pronounced improvement in these soil properties was observed in the S1W2-P treatment. Compared with S1W2, the S1W2-P treatment increased SOM, TN, and NO_3^--N contents by 27.23%, 11.03%, and 9.74%, respectively, while decreasing EC and NH_4^+-N content by 18.20% and 32.13%, respectively.

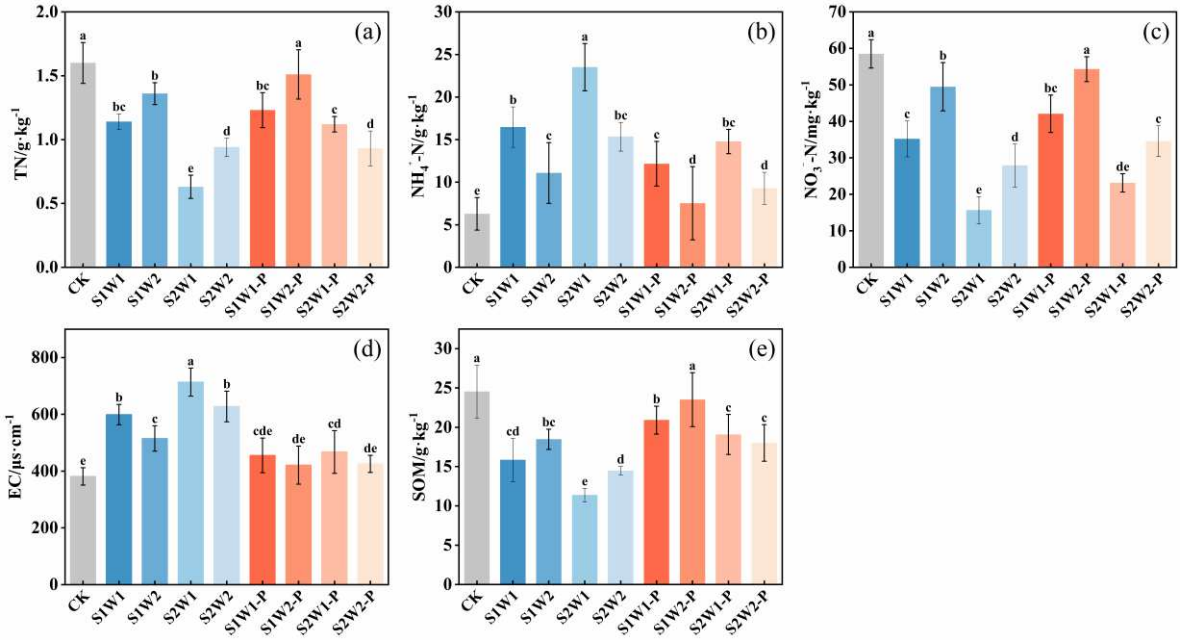


Fig. 2 Changes in soil physicochemical indicators under different treatments (a total nitrogen; b ammonium nitrogen; c nitrate nitrogen; d electrical conductivity; e soil organic matter). Different lowercase letters above bars indicate significant differences among treatments, $p < 0.05$.

3.2.2 Soil enzyme activity

Water and salt stress significantly inhibited soil enzyme activity, and the influence of soil

salinity stress on soil enzyme activity was more significant than that of water stress (Fig. 3). Compared with the CK, the average activities of urease, GS, NR, and nitrogenase in the S1W1, S1W2, S2W1, and S2W2 treatments decreased by 33.28%, 39.42%, 49.63%, and 40.98%, respectively.

Compared with the corresponding treatments without *B. subtilis* application, the treatments with *B. subtilis* application increased the average activities of urease, GS, NR and nitrogenase by 19.59%, 33.94%, 31.91% and 63.24% respectively. Among them, the activity of soil nitrogen metabolism enzymes (urease, GS, NR and nitrogenase) in the S2W1-P treatment was on average 65.56% higher than that in the S2W1 treatment; while the activity of soil nitrogen metabolism enzymes in the S2W2-P treatment was on average 45.53% higher than that in the S2W2 treatment. This indicates that *B. subtilis* application had a better promoting effect on the activities of soil nitrogen metabolism enzymes in higher salt and lower water environments.

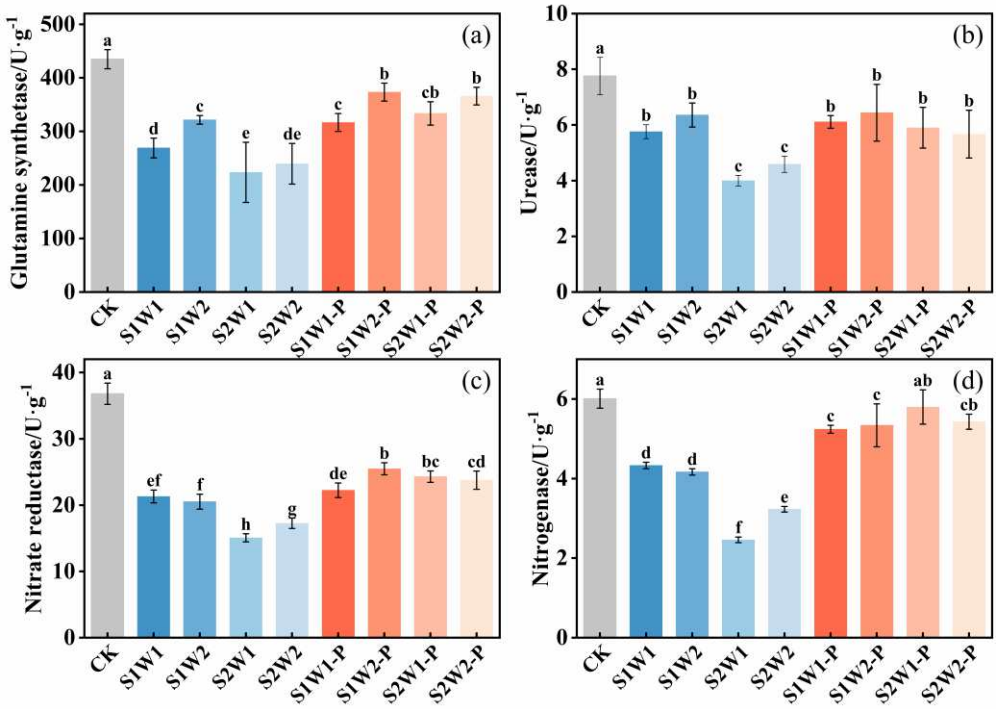


Fig. 3 Changes in soil enzyme activities under different treatments (a glutamine synthetase activity; b urease activity; c nitrate reductase activity; d nitrogenase activity). Different lowercase letters above bars indicate significant differences among treatments, $p < 0.05$.

3.3 Effects of *Bacillus subtilis* application on soil microbial community structure under water and salt stress

The composition and structure of soil bacterial communities are important indicators for evaluating soil ecosystem function and structure, and can reflect soil quality and productivity [35].

At the phylum level, *Proteobacteria*, *Acidobacteriota*, *Actinobacteriota*, and *Gemmatimonadota* were the dominant bacterial phyla across all treatments, accounting for 80.27-85.58% of the total relative abundance of the top 10 phyla (Fig. 4a). Compared to the CK treatment, the relative abundance of *Gemmatimonadota* in the treatments with and without *B. subtilis* decreased by an average of 4.30% and 8.44%, respectively. However, compared to the CK treatment, the relative abundance of *Chloroflexota* and *Gemmatimonadota* in the S2W2-P treatment increased by 45.84% and 11.25%, respectively. In addition, Proteobacteria and Bacteroidota showed increasing trends in some B. subtilis-added treatments compared with their corresponding non-inoculated treatments.

At the order level, *Gemmatimonadales*, *Vicinamibacterales*, and *Sphingomonadales* were the dominant bacterial orders, accounting for 49.53%–55.68% of the total relative abundance of the top 10 orders (Fig. 4b). *B. subtilis* application increased the relative abundances of *Gemmatimonadales* and *Vicinamibacterales* in some treatments under water and salt stress. Compared with the CK treatment, the relative abundances of *Gemmatimonadales* and *Vicinamibacterales* in the S2W2-P treatment increased by 5.84% and 26.53%, respectively. These results indicate that water and salt stress reduced the relative abundance of some adaptive bacterial taxa, higher soil water availability was associated with greater relative abundance of these taxa under high salinity conditions.

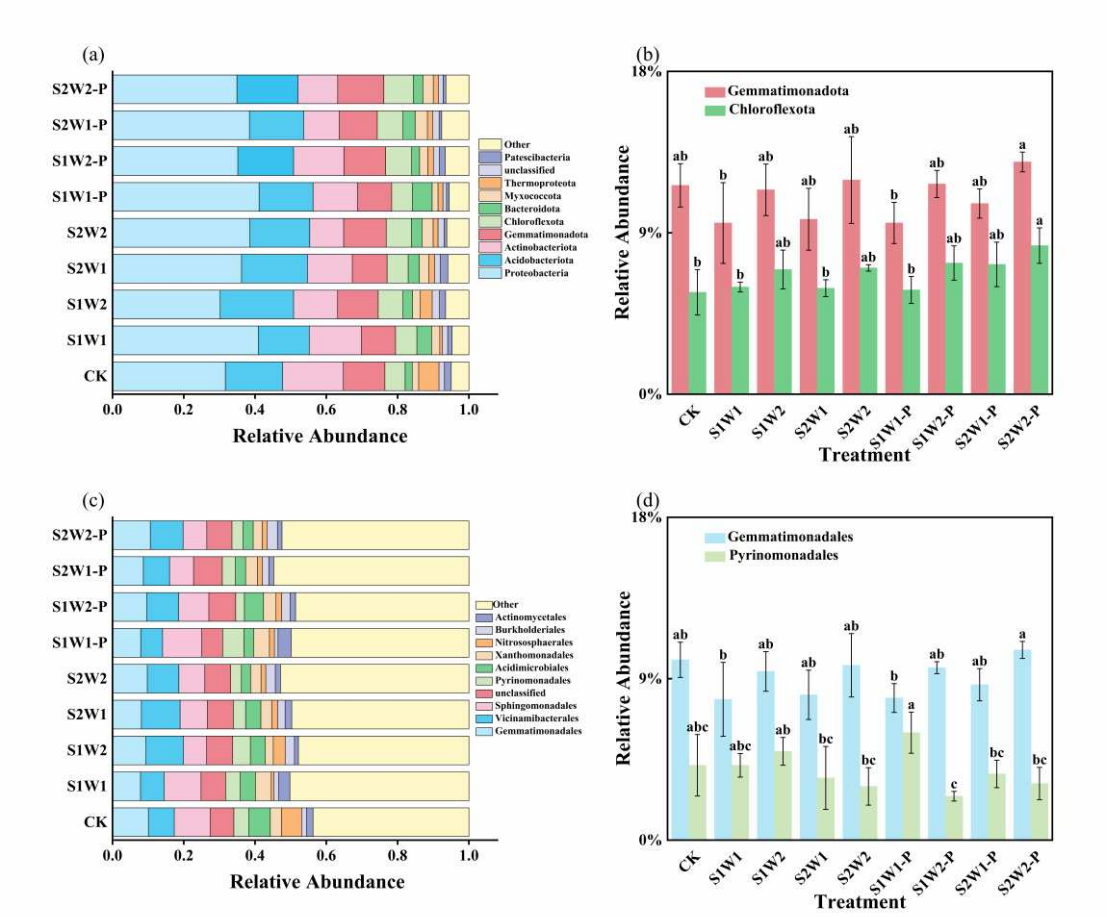


Fig. 4 Relative abundance of the top 10 bacterial taxa under different treatments (a phylum level; b order level).

There were no significant differences in soil bacterial community α-diversity among treatments ($P > 0.05$), although the application of *B. subtilis* tended to decrease soil bacterial community α-diversity (Fig. 5a, b). Principal coordinate analysis (PCoA)based on weighted UniFrac distances showed that the first two principal coordinates explained 49.2% of the total variation in bacterial community structure (Fig. 5c). Different treatments showed partial spatial separation. The S2W1-P and S2W2-P treatments were located farther from the CK treatment and showed clear separation from S2W1 and S2W2, respectively. These results suggest that high salinity altered the bacterial community structure, and the application of *B. subtilis* did not mitigate this effect.

RDA analysis showed that soil physicochemical properties and enzyme activities significantly explained rhizosphere bacterial community structure (($F = 6.1$, $P = 0.002$), accounting for 66.4% of the variation (Fig. 5d). Among these factors, NR, TN, EC, NO_3^--N, and

NH_4^+-N had significant effects on rhizosphere bacterial community structure ($P < 0.05$), whereas the other soil environmental factors had no significant effects ($P \geq 0.05$). These results indicate that NR, TN, EC, NO_3^--N, and NH_4^+-N were the key factors driving the observed differences in rhizosphere bacterial community structure.

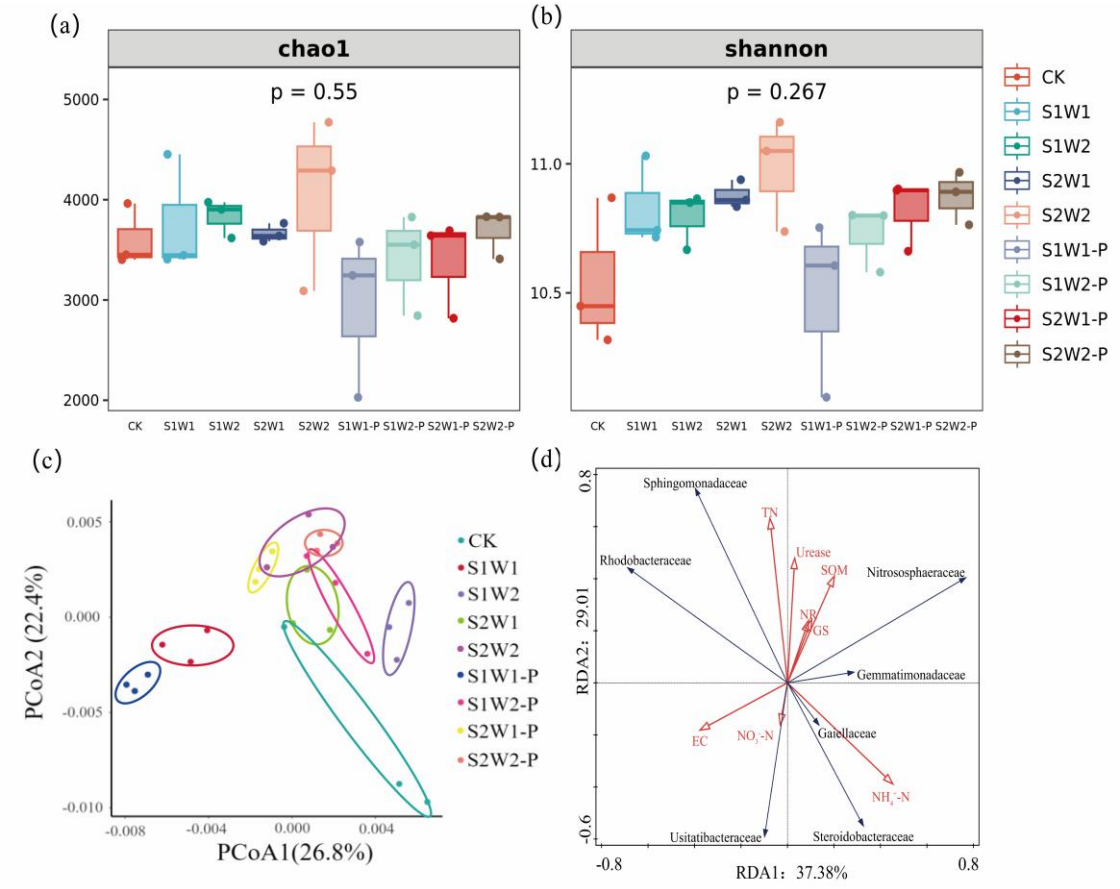


Fig. 5 Bacterial community diversity, structure, and associations with environmental variables under different treatments: (a) Chao1 index; (b) Shannon index; (c) principal coordinate analysis based on weighted Uni-Frac distance; and (d) redundancy analysis showing associations between bacterial community composition and environmental variables.

3.4 Integrated associations among inoculation, rhizosphere properties, and alfalfa growth

Fig. 6 shows the correlation analysis among soil enzyme activities, soil physicochemical properties, and alfalfa growth indicators. In the treatments without *B. subtilis*, GS and SOM were significantly negatively correlated with EC ($P < 0.05$), and nitrogenase was significantly negatively correlated with TN ($P < 0.05$), whereas dry weight was significantly positively correlated with urease. In the treatments with the application of *B. subtilis*, the correlations between soil enzyme activities and soil physicochemical properties were strengthened and shifted toward positive relationships. Specifically, the correlation coefficients of urease, NR, and nitrogenase

activities with SOM, TN, and NO_3^--N increased, and nitrogenase was extremely significantly positively correlated with TN ($P < 0.001$).

PLS-SEM was used to further investigate the regulatory pathways by which the application of *B. subtilis* affected the rhizosphere soil environment and alfalfa growth (Fig. 7). The model goodness of fit was 0.681, indicating that the overall prediction performance of the model was relatively accurate. Path analysis showed that the application of *B. subtilis* significantly enhanced soil enzyme activities related to nitrogen cycling (path coefficient = 0.796, $P < 0.001$) and reduced soil salinity (path coefficient = −0.782, $P < 0.01$). Meanwhile, *B. subtilis* application had a positive but non-significant direct effect on soil nutrient content (path coefficient = 0.199, $P \geq 0.05$). Soil nutrient content had a highly significant positive effect on alfalfa growth (path coefficient = 0.923, $P < 0.001$). Overall, the application of *B. subtilis* promoted alfalfa growth mainly by alleviating salt stress, enhancing soil enzyme activities related to nitrogen cycling, and improving soil nutrient availability, with soil nutrient content acting as a key mediating factor.

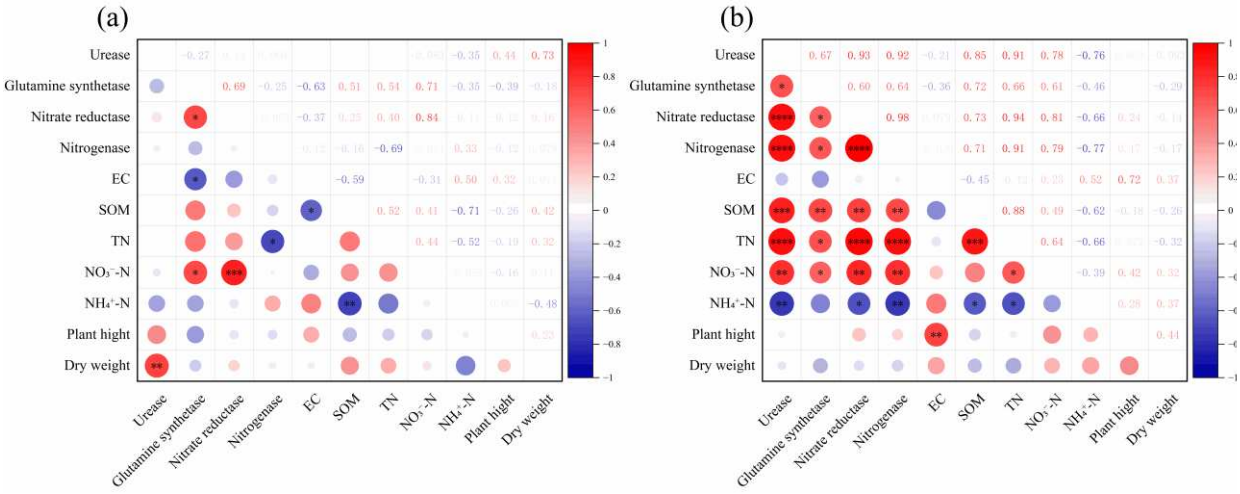


Fig. 6 Correlation heat maps of soil enzymatic activity, soil properties and alfalfa growth characteristics. without Bacillus subtilis treatment (a), with Bacillus subtilis treatment (b) *** $P<0.001$, ** $P<0.01$ and * $P<0.05$.

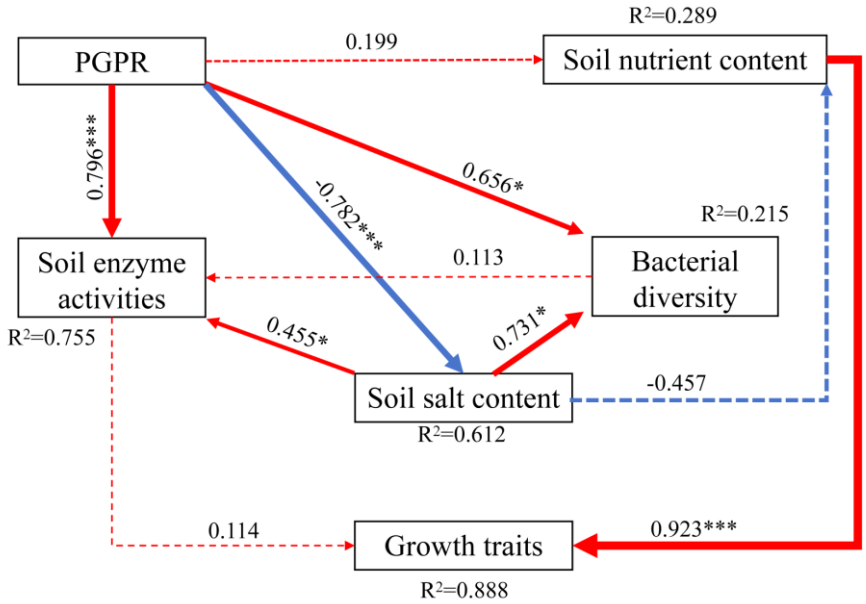


Fig. 7 Structural equation model for the associations influencing alfalfa growth. Note: * indicates P <0.05, ** indicates P <0.01, *** indicates P <0.001; solid lines represent significant relationships between the two indicators, while dashed lines indicate non-significant relationships. The numbers and line thickness on the arrows represent the path coefficients, and R^2 indicates the model's explanatory power.

3.5 Comprehensive Evaluation of Plant–Soil System Health

Fig. 8 shows the comprehensive evaluation of alfalfa growth physiology and soil health under different treatments. The GPI and SQI values in the treatments with the application of *B. subtilis* were significantly higher than those in the corresponding treatments without *B. subtilis* ($P < 0.05$), with average increases of 0.336 and 0.206, respectively. Among the treatments under water and salt stress, S1W2-P had the highest GPI, SQI, and PSI values, reaching 0.829, 0.734, and 0.781, respectively, and ranked second overall after CK. In contrast, S2W1 had the lowest GPI and PSI values, at 0.064 and 0.250, respectively.

The comprehensive evaluation showed that the application of *B. subtilis* increased the PSI values by 0.072–0.356 compared with the treatments without *B. subtilis*. Among these treatments, S1W2-P had a PSI value of 0.781, ranking second only to CK, whereas S2W1 had the lowest PSI value, at 0.250, and the greatest increase was observed in S1W2-P, with the PSI value being 0.356 higher than that in S1W2. Under the 4.5 $g \cdot kg^{-1}$ salt treatment, PGPR still had a certain alleviating effect, but its overall promoting effect on growth and soil improvement declined.

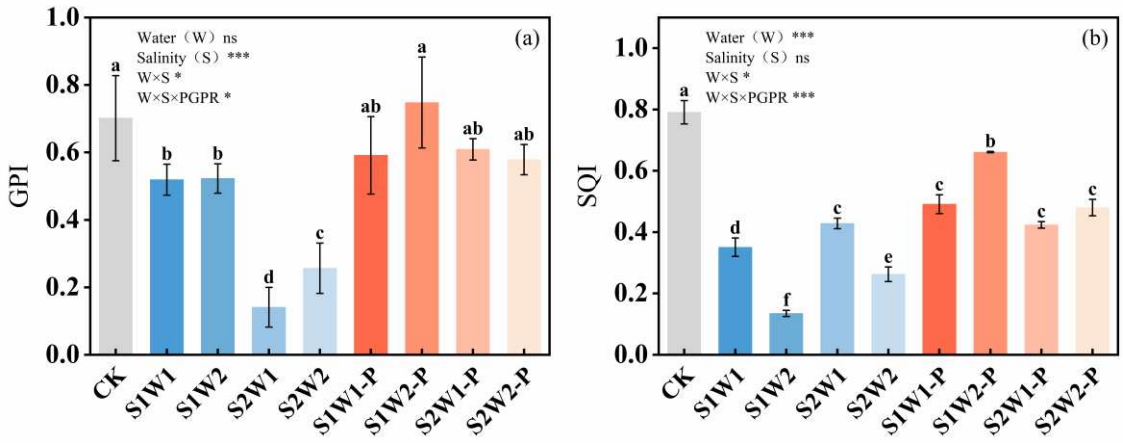


Fig. 8 Effects of Bacillus subtilis application under water and salt stress on the growth-physiology index and soil quality index of alfalfa-soil system (a growth-physiology index, GPI, b soil quality index, SQI). Values are means ± standard errors (n = 3). Different lowercase letters abov bars indicate significant differences among treatments ($P < 0.05$).

Table 2. Comprehensive Evaluation of Alfalfa and Soil Health

Treatment	GPI	SQI	PSI	Rank
CK	0.791	0.886	0.838	1
S1W1	0.542	0.454	0.498	6
S1W2	0.554	0.296	0.425	7
S2W1	0.064	0.436	0.250	9
S2W2	0.217	0.374	0.296	8
S1W1-P	0.641	0.499	0.570	5
S1W2-P	0.829	0.734	0.781	2
S2W1-P	0.642	0.540	0.591	4
S2W2-P	0.607	0.611	0.609	3

4 Discussion

4.1. Effect of *Bacillus subtilis* on alfalfa physiology and growth under water and salt stress

The antioxidant enzyme system is an important defense mechanism by which plants resist environmental stress. SOD and CAT are key antioxidant enzymes involved in scavenging excess ROS and alleviating membrane lipid peroxidation damage, and they are also important indicators of plant stress resistance. Increased salinity and water deficit can induce ROS accumulation, and when antioxidant defenses are insufficient to remove excess ROS, membrane lipid peroxidation damage is aggravated [36, 37]. At the same time, plants usually maintain cellular osmotic balance by accumulating osmotic regulators such as proline, although excessive accumulation also indicates that plants are subjected to severe stress [38, 39]. MDA is an important product of

membrane lipid peroxidation, and its accumulation generally reflects the extent of oxidative damage to cell membranes. Previous studies have shown that water deficit and salt stress can disrupt ROS homeostasis in plants and induce the accumulation of MDA and osmotic adjustment substances [27, 37-39]. In this study, water and salt stress increased SOD and CAT activities, as well as MDA and proline contents, in alfalfa. This indicates that alfalfa activated antioxidant defense and osmotic adjustment mechanisms in response to water and salt stress. However, the simultaneous increase in MDA content also suggests that membrane lipid peroxidation was aggravated under stress conditions. When salinity reached 4.5 $g \cdot kg^{-1}$, compared with the CK treatment, plant height, fresh weight, and dry weight under water and salt stress decreased by 7.58%, 35.85%, and 47.42%, respectively. Notably, dry weight under S2W1 treatment decreased significantly by 79.01% relative to CK, indicating that the combined effects of high salinity and water deficit markedly inhibited photosynthate accumulation and dry weight accumulation in alfalfa, thereby reducing plant growth.

Previous studies have shown that *B. subtilis* can improve the physiological and metabolic status of crops under salt stress by increasing SOD, POD, and CAT activities and decreasing MDA and proline contents [27, 40-42]. Similar results were obtained in the present study. Under the same water and salt conditions, the application of *B. subtilis* improved alfalfa stress resistance mainly by increasing CAT activity and decreasing MDA and proline contents. Compared with the corresponding treatments without *B. subtilis*, the application of *B. subtilis* increased CAT activity by 10.58–27.61%, while MDA and proline contents decreased by 3.45–27.13% and 14.26–29.75%, respectively. In particular, compared with the S2W1 treatment, CAT activity and dry weight in the S2W1-P treatment increased by 27.81% and 188.43%, respectively. Although the largest relative increase in dry weight occurred under S2W1, this treatment had a low baseline growth level under severe stress. Therefore, the large percentage increase should not be interpreted as greater overall PGPR effectiveness under high salinity. The integrated evaluation instead indicated that the overall benefit was greatest under moderate salinity and adequate soil water availability. This result is similar to that reported for maize by Shabaan et al.[43]. However, the present study also found that when salinity reached 4.5 g kg^{-1}, SOD activity in alfalfa decreased in the treatments with the application of *B. subtilis*. This phenomenon may be explained by the fact that under high-salinity stress, plants accumulate large amounts of superoxide anions (O_2^-), which induces SOD synthesis

449 to rapidly remove excess O_2^- and maintain ROS homeostasis [44]. Based on previous studies,
450 extracellular polysaccharides secreted by *B. subtilis* may restrict Na^+ influx and alleviate ion
451 toxicity, which may partly explain the decreased SOD activity under high salinity in this study
452 [41]. Moreover, *B. subtilis* can simultaneously activate multiple antioxidant enzymes. For example,
453 enhanced CAT activity may rapidly remove H_2O_2 and reduce oxidative damage. This is also
454 consistent with Ma et al. [45], who found that ACC deaminase activity in some strains decreased
455 under high salinity, suggesting that excessive salinity can weaken the growth-promoting metabolic
456 function of PGPR.

457 4.2. Effect of *Bacillus subtilis* on soil properties, soil enzyme activity and bacterial communities under
458 water and salt stress

459 PGPR effects under water and salinity stress are closely associated with changes in the
460 rhizosphere soil environment and nutrient availability. Sun et al.[46] also suggested that
461 improvement of the rhizosphere soil environment is an important basis for the growth-promoting
462 effect of PGPR. Rhizosphere microbial communities are an important link between changes in the
463 soil environment and plant growth responses. In this study, *Proteobacteria*, *Acidobacteriota*,
464 *Actinobacteriota*, and *Gemmatimonadota* collectively constituted the predominant bacterial phyla
465 in rhizosphere soil across treatments. This finding is consistent with Bi et al.[27], who reported
466 that *B. subtilis* altered the composition and structure of cotton rhizosphere bacterial communities
467 under water and salt stress.

468 Previous studies have shown that salinity or drought can alter rhizosphere bacterial
469 community structure and weaken the stability of microbial groups associated with nutrient cycling
470 and stress adaptation. However, the application of PGPR does not necessarily increase species
471 richness, but may maintain rhizosphere ecological processes by regulating the proportions of
472 dominant functional groups [47-49]. There were no significant differences in bacterial alpha
473 diversity among treatments, although a decreasing tendency was observed following *B. subtilis*
474 application. Changes in bacterial community composition were accompanied by changes in soil
475 nutrient status. In contrast, previous studies have shown that salt stress can weaken available
476 nitrogen supply by reducing soil water potential, inhibiting microbial activity, and altering nitrogen
477 transformation processes [50]. These changes indicate a shift in rhizosphere inorganic N status
478 under combined water and salinity stress. Compared with CK, soil NH_4^+-N increased by an

479 average of 164.67%, whereas NO_3^--N decreased by 45.20% under water and salt stress without *B.*
480 *subtilis*.

481 Previous studies have reported that PGPR application can increase soil enzyme activities
482 under salinity stress [15, 23, 51]. In this study, *B. subtilis* application enhanced soil enzyme
483 activities. Compared with the corresponding treatments without *B. subtilis*, urease, NR, GS, and
484 nitrogenase activities increased by 19.59%, 33.94%, 31.91%, and 63.24% on average, respectively.
485 Bi et al. [50] similarly reported that *B. subtilis* improved crop physiology and growth by optimizing
486 soil water, salt, and nitrogen conditions.

487 The PLS SEM analysis showed that *B. subtilis* application was negatively associated with
488 soil salinity and positively associated with nitrogen related enzyme activities, whereas soil nutrient
489 status was strongly positively associated with alfalfa growth. Similar responses have been reported
490 in previous studies, which showed that PGPR application can enhance soil enzyme activities and
491 improve rhizosphere nutrient conditions under salinity stress [26, 51]. In the present study, the
492 direct association between B. subtilis application and soil nutrient status was positive but not
493 significant. These results suggest that lower soil salinity, greater nitrogen related enzyme activities,
494 and improved nutrient availability were closely associated with the positive growth responses
495 observed following *B. subtilis* application. These relationships should be interpreted as statistical
496 associations rather than direct evidence of causal pathways.

497 **5 Conclusions**

498 This study demonstrated that *Bacillus subtilis* inoculation improved alfalfa growth under
499 combined water and salinity stress, although the magnitude of its beneficial effect depended on
500 soil water availability and salinity severity. Inoculation increased alfalfa dry biomass and CAT
501 activity while reducing proline and MDA accumulation, indicating alleviation of osmotic stress
502 and membrane lipid peroxidation. These plant responses were accompanied by lower
503 rhizosphere EC, improved SOM, TN, and NO_3^--N availability, and enhanced activities of
504 nitrogen-related soil enzymes. Changes in bacterial community structure were closely associated
505 with rhizosphere salinity, nitrogen availability, and nitrate reductase activity, suggesting

coordinated plant–rhizosphere responses to inoculation rather than a simple increase in bacterial diversity. The most favorable overall response occurred at 3.0 g kg^{-1} salinity and 70-85% field capacity, whereas the benefit of inoculation was constrained under the higher salinity level. Therefore, coordinating *B. subtilis* application with appropriate water management may provide an effective biological strategy for improving alfalfa performance in salt-affected environments.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Not applicable.

Authors' information

Not applicable.

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Author contributions

DT conceptualized this study. CH, ZQ, DL and PT designed the experiment. BY and RL developed the experimental methodology. XC conducted the experiment, performed the data analysis, and wrote the original draft of the manuscript. CH reviewed and edited the manuscript. DT supervised the research. WL and NC provided research resources. CH, ZQ and DT acquired funding for this study. All authors read and approved the final manuscript.

Data availability

The raw 16S rRNA amplicon sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1510339. The remaining datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable reques.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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