Journal of Soil Science and Plant Nutrition (2024) 24:4033–4048
https://doi.org/10.1007/s42729-024-01828-4

ORIGINAL PAPER

Effects of Rhizosphere Microorganisms Associated with *Suaeda Salsa* on the Growth and Salt Stress Resistance of Alfalfa

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Received: 23 October 2023 / Accepted: 14 May 2024 / Published online: 21 May 2024

Abstract
Plant growth-promoting rhizobacteria (PGPR) are soil microorganisms that interact with plants to enhance growth and development. In the past, PGPR screening has primarily focused on isolates from the rhizosphere of the target plant, with salt-tolerant strains from saline alkali soil typically utilized for soil improvement or as backup strains, rather than as PGPR. Limited research has been conducted on the efficacy of selected salt-tolerant strains in promoting the rhizosphere of alfalfa (*Medicago sativa* L.). This study employed the salt-tolerant strain *Bacillus tropicus* (YJ33) as the subject of investigation in the rhizosphere of *Suaeda salsa*, utilizing indoor pot experiments to comprehensively investigate its influence on alfalfa growth. The findings shed light on the role and impact of microorganisms in saline alkali soil on the growth of non-native plant species such as alfalfa. The application of YJ33 resulted in a significant enhancement in biomass production ($p < 0.05$), plant height ($p < 0.05$), and antioxidant enzyme activity ($p < 0.05$) of alfalfa, while also leading to a significant reduction in malondialdehyde (MDA) content ($p < 0.05$) and promoting overall growth under salt stress conditions. Furthermore, inoculation with the strain significantly elevated the crude protein content($p < 0.05$) of alfalfa and facilitated phosphorus absorption ($p < 0.05$).Additionally, this study demonstrated that inoculation with strain significantly increased the levels of soil nitrate nitrogen, ammonium nitrogen, and available phosphorus. Furthermore, the introduction of the inoculated strain had a notable impact on the abundance of Firmicutes, Proteobacteria, and Actinobacteria. These alterations in phyla closely mirrored the shifts observed in soil characteristics and alfalfa physiology following inoculation. The salt-tolerant and growth-promoting bacteria identified in this study have been found to enhance the stress resistance of alfalfa in saline-alkali conditions, as well as facilitate plant phosphorus uptake, improve soil microbial conditions, and optimize the utilization of nitrogen and phosphorus elements.

Keywords PGPR · *Bacillus tropicus* · Salt-tolerant microorganisms · Soil bacterial community · Alfalfa

1 Introduction

Soil salinization is a pressing global environmental issue that is currently being confronted on a worldwide scale (Abiala et al. 2018). The total expanse of saline alkali soil across the globe is estimated to be approximately 954.38 million hm^2 (Wang et al. 2003; Yang et al. 2023), with China standing out as one of the nation's grappling with severe salinization, encompassing an area of about 99.13 million hm^2 (Mao et al. 2014). Saline alkali soil is distinguished by elevated soil salinity levels, inadequate soil structure, diminished vegetation cover, and reduced crop yields, thus posing a significant challenge on a global scale. Therefore, some plants are difficult to survive under these conditions (Wang et al. 2010). The saline alkali soil in Shandong Province mainly includes

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saline alkali soil in the Huanghuaihai Plain and coastal saline alkali soil (Xu et al. 2021). The Yellow River Delta, encompassing approximately 500,000 hm^2, features typical coastal saline alkali soil and serves as a significant reserve land resource in China (Yang and Sun 2020). However, the fertility of the soil is compromised by severe salinization, resulting in low and unstable crop yields (Li et al. 2021b). The development and utilization of moderate to mild saline soils hold significant importance for enhancing agricultural production and improving the ecological environment (Xu et al. 2021). Given the escalating pressures on global population, food security, resources, and environmental sustainability, identifying and effectively utilizing cultivated land reserve resources is crucial for maintaining dynamic equilibrium. Consequently, the development and utilization of saline alkali soil emerges as a vital and effective strategy for addressing contemporary challenges and harnessing the potential of saline alkali soil. Numerous studies have demonstrated the ability of PGPR to enhance plant root growth and biomass under salt stress conditions (Cai et al. 2021). Additionally, research has shown that the application of PGPR preparations containing 1-aminocyclopropane-1-carboxylate deaminase activity in saline alkali soil mitigate the inhibitory effects of high salinity on root growth by reducing ethylene concentration in plants (Nadeem et al. 2010). Furthermore, Barnawal et al. (2017) have reported that PGPR can elevate the levels of indole-3-acetic acid (IAA) and abscisic acid (ABA) in wheat plants, thereby enhancing their resistance to salt and alkali stress while promoting overall growth and development. The utilization of salt-tolerant plants in the amelioration of saline alkali soil has the potential to enhance ecosystem stability (Shahbaz and Ashraf 2013). However, the time-consuming nature of breeding plant varieties with desirable salt tolerance poses challenges to this approach. Furthermore, the advancement of more efficient, widespread, and easily controllable microbial remediation technologies for saline alkali soil represents a crucial avenue for achieving soil improvement. For instance, the incorporation of microorganisms capable with nitrogen fixation and phosphorus solubilization can effectively serve the goal of enhancing alkali soil quality. Prior research has demonstrated that endophytic or rhizosphere microorganisms present in halophytes can improve plant salt tolerance (Ibort et al. 2017). Additionally, plant growth-promoting rhizobacteria (PGPR) obtained from salt-stressed tomato root bulbs have been found to decrease Na^+ levels in tomato plants, enhance enzyme activity in both tomato plants and soil, and stimulate tomato growth in the presence salt stress (Islam et al. 2015). Some important functional microorganisms, include Actinobacteria, Actinomyces, arbuscular mycorrhizal fungi (AMF) (Lin et al. 2017), and mold (Babu et al. 2014), have been used to improve saline alkali soil. A previous study screened highly saline alkali-tolerant *Trichoderma*, which not only lowers the pH and electrical conductivity (EC) of red soil but also stimulates plant growth (Anam et al. 2019). The aforementioned microbial processes have the potential to enhance the viability and longevity of plant life in saline-alkali soil to a certain degree, thereby contributing significantly to the sustained amelioration of saline-alkali soil conditions, the restoration of soil properties, and the reconstruction of the ecological environment in saline-alkali soil. Despite research indicating that halophytic rhizosphere microorganisms can enhance the salt tolerance of non-native crops, the precise mechanisms underlying their effects remain uncertain (Wang et al. 2022b).

Planting salt-tolerant plants in saline alkali soil has been shown to enhance the quality of the soil, increase soil nutrient content and increase the diversity and abundance of microorganisms in saline alkali soil (Yang et al. 2019a, 2022). Saline-tolerant plants allocate a significant portion of photosynthetic products to the root system, with over 40% of these materials being released into the rhizosphere through root exudates. This carbon source provided by the plants supports the growth and activity of rhizosphere microorganisms, which in turn mineralize inorganic nutrients in the soil for the benefit of plant growth and development (Lynch and Whipps 1990). Forage is frequently selected as the primary material for expeditious soil enhancement due to its rapid growth, substantial biomass yield, and robust regenerative capacity (Paula et al. 2020). Alfalfa (*Medicago sativa* L.), a superior perennial leguminous forage characterized by its considerable production capacity, extensive utility, exceptional nutritional quality, and vigorous nodule nitrogen fixation, represents a crop with notable salt tolerance that can serve as a beneficial green manure for soil enhancement and a proficient feed source for livestock husbandry (Yi et al. 2018). The act of planting alfalfa has been shown to have a multifaceted impact on agricultural systems, including enhancing soil fertility, promoting vegetation restoration, and supporting consistent high grain yields. Additionally, alfalfa cultivation serves important ecological functions by aiding in soil and water conservation, mitigating secondary salinization of farmland, and enhancing overall soil health (Yang et al. 2019b, 2020). *Suaeda salsa*, a prevalent halophyte in coastal saline alkali regions, exhibits a distinct microbial community in its rhizosphere soil compared to conventional crops like wheat, as demonstrated by recent studies (Wei et al. 2020). This observation leads us to hypothesize that the rhizosphere microorganisms associated with *Suaeda salsa* may confer enhanced stress resistance to alfalfa in saline alkali environments. The objective of this research was to investigate the impact of screening bacteria from the rhizosphere of *Suaeda salsa* on the growth,

physiology, and salt tolerance of alfalfa post inoculation, assess the influence of inoculation with this strain on soil properties and the microbial community of alfalfa, and analyze the correlation between inoculation with this strain and soil properties, soil microbial community, and physiological attributes of alfalfa.

2 Materials and methods

2.1 The Identification of Bacterial Strains and the Construction of Phylogenetic Trees

Soil samples were collected from the rhizosphere soil of *Suaeda salsa* in Wudi County, Shandong Province (37°54′60″E, 117°57′33″N). Following collection, the samples were sieved through a 2 mm mesh, placed in pre-sterilized bags, and sealed. Subsequently, they were promptly stored in a container with dry ice and transported to the laboratory for preservation at 4 ℃. To prepare the soil suspension, 100 mL of distilled water was added to a conical flask along with 45 mL of the soil sample. Seven glass beads were then included in the flask, which was subsequently sterilized in an autoclave. Add 5 g of soil sample to a sterilized conical flask placed on an ultra-clean workbench, and agitate at 28 °C and 180 revolutions per minute for a duration of 20 min. Subsequently, dilute the resulting soil suspension to concentrations ranging from 10^{-2} g/mL to 10^{-6} g/mL by combining 1 mL of soil suspension with 9 mL of sterile water. Extract 0.2 mL of the diluted solutions with concentrations of 10^{-3} g/mL, 10^{-4} g/mL, and 10^{-5} g/mL, and inoculate beef paste tryptone medium plates with a pH of 8.0. Incubate the plates at 28 °C, and isolate distinct strains from each plate using the streak method on beef paste tryptone medium supplemented with 200 mM NaCl. Following purification, a portion of the isolated strain will be transferred to a slanted preservation culture and maintained at 4 ℃, while another portion will be preserved in a 50% glycerol tube at -80℃. Morphological identification of the strain involved the utilization of Gram staining and negative staining techniques. An Ezup columnar bacterial genomic DNA extraction kit was used for DNA extraction. Amplification and sequencing of 16SrDNA genes in the bacterial genome were performed using universal primers 27 F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGATT-3'). The amplification reaction solution was 25 μL, containing 9.5 μL of ddH_2O, 12.5 μL of 1×T3 Mix, 1 μL of each primer, and 1 μL of the template solution. The PCR reaction conditions were as follows: denaturation at 95 °C for 5 min, then 30 cycles containing heat denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension for 90 s at 72 °C, and final extension for 10 min at 72 °C. The obtained strain sequences were submitted to the GenBank database established by the NCBI. BLAST was used to search for homologous genes, and the neighbor-joining method was used to construct a phylogenetic tree using MEGA10.0 software (Paeng and Park 2019). Through examination of the strain's physicochemical characteristics and phylogenetic analysis of its 16SrDNA sequence, the strain we obtained (namely YJ33 in this study) was conclusively identified as *Bacillus tropicus* (GenBank accession number: SUB14346684), as illustrated in Fig. S1.

2.2 Salt Tolerance and Growth Promoting Characteristics of Strains

Prepare the identified strains by creating a seed solution, then incorporate 2% of this solution into beef tryptone liquid culture media containing varying salt concentrations. Incubate the mixture at 28 ℃ and 180 revolutions per minute for a duration of 24 h to assess the OD_{600} value and ascertain the strain's salt tolerance. Fig. S2 demonstrates that the strain morphology of YJ33, which is capable of surviving in a salt concentration of 1000mmol/L and exhibits the highest growth rate until a salt concentration of 200mmol/L.

Inoculate 1 ml of bacterial suspension into NBRIP inorganic phosphorus liquid medium with each strain set up in triplicate, using non-inoculation as the control. The cultures should be shaken and incubated at 28 ℃ and 180 r/min for a period of 10 days. Following incubation, the pH of the culture medium should be measured, and the cultures centrifuged at 4 ℃ and 10,000 r/min for 15 min. Subsequently, 5 mL of the supernatant should be transferred to a 150 mL triangular flask and mixed with 50 mL of 0.5 mol/L sodium bicarbonate extract. A spoonful of phosphorus-free activated carbon powder should be added, and the flask sealed and shaken on a shaking bed for 30 min. The mixture should then be filtered using phosphorus-free filter paper. Transfer 10 mL of filtrate into a 50 mL volumetric flask and add 5 mL of molybdenum antimony anti chromogenic agent. After thorough shaking, adjust the volume to the mark and allow the solution to stand at room temperature for 30 min before comparing the color at a wavelength of 700 nm. Utilizing the liquid culture molybdenum antimony colorimetric method, the phosphorus solubilization ability of the tested phosphorus solubilizing bacteria was assessed. The phosphorus standard curve was determined to be $y = 0.6575x + 0.0724$ ($R^2 = 0.9952$), illustrating the relationship between phosphorus concentration and phosphorus solubilization of this particular strain as depicted in Fig. S3. Strain YJ33 demonstrated a notable capacity for phosphorus solubilization in comparison to the control group, as evidenced by statistical significance ($P < 0.05$).

2.3 Pot Experiment

Prior to commencing the experiment, germination experiments were conducted on multiple sequenced strains to identify those exhibiting salt tolerance and growth-promoting functions. *Bacillus tropicus* YJ33 was chosen for further potted experiments, which were carried out in an artificial climate chamber from start to finish. The *Bacillus tropicus* strain YJ33 was utilized to inoculate a beef tryptone liquid culture medium within a sterile workbench environment, maintained at a temperature of 28 °C and an agitation rate of 180 rotations per minute for a duration of 24 h. Subsequently, the culture was subjected to centrifugation at 8000 rotations per minute for 10 min at a temperature of 4 °C. The resulting supernatant was discarded, and the bacterial cells were harvested. These cells were then resuspended in sterile water to create a bacterial solution with an optical density at 600 nm (OD_{600}) value of 2.0 ± 0.01, which was prepared for future experimentation. The study utilized a two-factor randomized block design, incorporating four NaCl salt treatments (S1: 75 mmol/L, salt content 4.38‰; S2: 150 mmol/L, salt content 8.77‰; S3: 225 mmol/L, salt content 13.16‰; S4: 300 mmol/L, salt content 17.55‰)and two bacterial concentration treatments (inoculation treatment: 4×10^9 cfu/mL, non-inoculated treatment: 0 cfu/mL). In this design B denoted the inoculation treatment, while C represented the control treatment without inoculation. Each experimental treatment was replicated five times, resulting in a total of 40 treatments (Table S1).

Choose alfalfa seeds that are uniform in size, fully mature, plump, and free from insect damage. Lay them evenly on two layers of filter paper in a culture dish after disinfection treatment, saturate the filter paper with water, and transfer the dish to a culture box to facilitate germination. Alfalfa seeds were initially germinated in incubator, with selection criteria based on uniform size, maturity, and absence of insect damage. The growth conditions included a light-dark cycle of 16 h and 8 h, temperatures ranging from 22 to 28 °C, and humidity levels between 50% and 80%. Upon full unfolding of both cotyledons, the alfalfa seedlings were transplanted into plastic flower pots with dimensions of 8 cm in bottom diameter, 10 cm in top diameter, and a depth of 13 cm. In order to mitigate the impact of additional microorganisms on the experimental results, the soil mixture (composed of nutrient soil, vermiculite, and perlite in a ratio of 4:1:1) was thoroughly mixed and sterilize twice in a high-temperature sterilization pot set at 121 degrees Celsius, with each sterilization cycle lasting 20 min. Subsequently, the soil was subjected to varying concentrations of salt treatments. Following this, 50 mL of salt solution was evenly distributed into each pot, and once absorbed, uniform alfalfa seedlings displaying consistent growth were carefully transplanted. Each pot accommodated fifteen plants, and thinning was conducted once the first compound leaf of the plant seedlings had fully unfurled. The experimental group received treatment with 50 mL of *Bacillus tropicus* YJ33 bacterial agent with an optical density (OD_{600}) of 2.0 on days 7, 14, and 21 following the transfer of seedlings to flower pots for a duration of two weeks. In contrast, the control group was administered an equivalent volume of sterile water. Throughout the growth phase, watering was conducted using sterile water, and the moisture levels in each pot were monitored using a moisture analyzer (ZBS-SWEP) to ensure maintenance within the range of 20–40%. The flower pot's placement was altered on a weekly basis in order to mitigate discrepancies in lighting conditions across various positions, thereby minimizing errors.

2.4 Sampling and Measurement of Alfalfa Growth and Physiological Characteristics and Soil Characteristics

Sampling commences following a growth cycle lasting two months. The physiological and biochemical indicators, total biomass, plant height, and root length were assessed. Biomass quantification involved rinsing both aboveground and underground plant parts with tap water, blotting excess moisture with absorbent paper, placing the tissues in paper envelope bags, drying in a 105 °C oven for 30 min, baking at 65 °C until a constant weight was achieved, and subsequently determining the dry weight.

In the analysis of antioxidant enzymes, fresh plant leaf samples weighing 0.1 g were processed by grinding in a plant tissue grinder. The resulting plant material was then transferred to a 1.5 mL centrifuge tube, where 1 mL of extraction solution was added. The samples were homogenized in an ice bath and subsequently centrifuged at 4 °C for 10 min at a speed of 8000 revolutions per minute. The supernatant obtained was stored on ice for further analysis. Malondialdehyde (MDA) levels were determined using the thiobarbituric acid method as outlined by researcher, and superoxide dismutase (SOD) activity was assessed using the nitrogen blue tetrazole method (NBT) (Lou et al. 2018). Peroxidase (POD) activity was measured using the guaiacol method (Rao et al. 1997). Catalase activity was measured using the hydrogen peroxide decomposition method (Wang et al. 2009).

In order to ascertain the crude protein (CP) content, the crushed alfalfa sample underwent sieving through a 60 mesh sieve, followed by the extraction of a 10 mg portion of the sample using a tin foil boat. The total nitrogen content was subsequently determined utilizing an elemental analyzer (Elemental), and the resulting nitrogen content was then multiplied by a coefficient of 6.25 to derive the

crude protein content. The phosphorus content in alfalfa was determined by digesting crushed plant samples using the H_2SO_4-H_2O_2 method and analyzing them with a fully automatic flow analyzer. Soil total carbon (TC), total nitrogen (TN), and carbon nitrogen ratio (C/N) were measured using an elemental analyzer (Vario EL cube, Germany). Soil NH_4^+- N and NO_3^--N concentrations were determined using a Continuous Flow Analytical System (AutoAnalyzer 3, Germany). Phosphorus availability was assessed through the extraction of available phosphorus (AP) using the sodium bicarbonate molybdenum antimony colorimetry method, followed by quantification using an enzyme-linked immunosorbent assay.

2.5 Sample DNA Extraction

Rhizosphere soil DNA was extracted using the E.Z-N.A. method. A soil DNA kit, E.Z-N.A. ® The Soil DNA Kit DNA Extraction Kit (Omega, USA), was used to determine the quantity and quality of DNA according to the manufacturer's instructions using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) (Rodrigues et al. 2013).

2.6 PCR Amplification and Sequencing Library Construction

The V3-V4 region of the bacterial 16 S rRNA gene was amplified by utilizing the extracted DNA as a template and employing the upstream primer 338 F (5'-ACTCCTACGG GGCAG-3') and downstream primer 806R (5'-GACTACH-VGGGTWTCTAAT-3') (Liu et al. 2016). The NEXTFLEX Rapid DNA-Seq Kit was utilized for the construction of a library consisting of purified PCR products. This process involved the use of a connector link, magnetic beads for screening and removal of self-connected fragments, enrichment of library templates through PCR amplification, and recovery of PCR products using magnetic beads to obtain the final library. Detailed procedures for these steps can be found in the Supplementary Material.

2.7 Statistical Analysis

The SPSS 19.0 software were used to organize the obtained data and to conduct one-way and multivariate analysis of variance. The LSD multiple comparison method was used in the one-way analysis of variance, and the significance of differences was determined between each treatment. MOTHUR1.30.2 software was used to determine the α diversity index, including the Chao and Shannon diversity indices. The Wilcoxon rank sum test was used for inter-group difference analysis of alpha diversity. The principal coordinate analysis (PCoA) was used based on the Bray–Curtis distance algorithm (principal coordinate analysis) to test the similarity of microbial community structure between samples and combined with the PERMANOVA non-parametric test to determine whether the differences in microbial community structure between sample groups were significant (Yang et al. 2021). The R language package "Vegan" (vsesion 2.4.3) was used for RDA analysis, and PCA analysis was constrained by environmental factors. The spearman correlation coefficients between microorganisms, alfalfa physiology, and soil properties were calculated and plotted using the R package "Corrplot" (V.0.88). The structural equation model (SEM) was developed by AMOS (IBM SPSS AMOS23) using maximum likelihood estimation. This model was used to evaluate the effects of inoculated strains on microbial communities, soil properties, alfalfa physiology, and growth. The applicability of SEM was tested based on a non-significant chi-square test ($P > 0.05$) and approximate root mean square error (RMSEA) (Yang et al. 2023).

3 Results

3.1 Effect of Salt Content and Bacterial Concentration on Alfalfa Growth

In the context of BS1 treatment, alfalfa biomass reached its peak level (Fig. 1a). Under the same salt content treatment, the biomass of inoculated alfalfa was significantly higher than that of non-inoculated alfalfa ($P < 0.05$). Under the bacterial inoculation treatment, the total biomass showed a decreasing trend with an increase in salt content. The plant height showed a decreasing trend with an increase in salt content (Fig. 1b). Under the non-inoculated treatment, the lowest plant height of alfalfa was 9.63 cm when treated with the salt content S4 treatment (300 mmol/L). The plant height under salt content S1 treatment (75 mmol/L) was significantly higher than those of the other three salt content treatments ($P < 0.05$). In the inoculation treatment, the alfalfa plant height was highest under salt content S1 treatment (20.45 cm), which was 14.4%, 30.8%, and 43.9% higher than BS2, BS3, and BS4, respectively, and decreased with the increase in salt content (Fig. 1c). Figure 1d illustrates the morphological alterations in alfalfa subjected to various treatments, with the observed structural characteristics reflecting the plant's response to stress. Analysis of alfalfa growth following exposure to differing salt concentrations and bacterial inoculations revealed a pronounced inhibition in growth with increasing salt levels, evidenced by reduced plant height and the development of small, yellow leaves. Additionally, plants inoculated with *Bacillus tropicus* strain

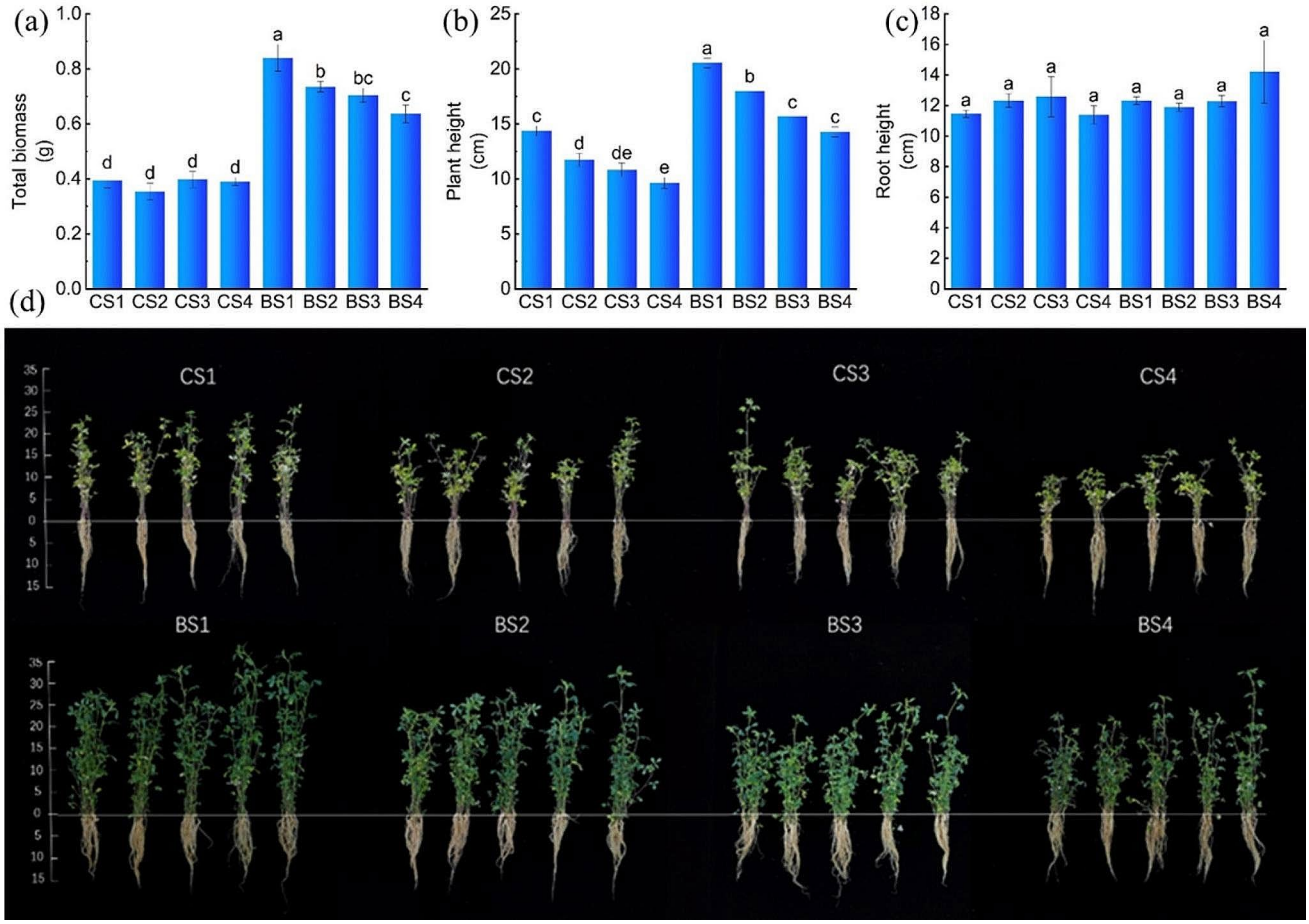

Fig. 1 The effects of inoculation and non-inoculation under different salt concentrations on the biomass (**a**), plant height (**b**), root length (**c**) and growth status of alfalfa (**d**). Values with different letters are significantly different at $p < 0.05$ by variance analysis

YJ33 exhibited enhanced growth compared to non-inoculated counterparts.

In order to assess the impact of salt content and bacterial concentration on alfalfa plants, various parameters including alfalfa antioxidant enzyme activity were measured. In the non-inoculated treatment, the MDA content exhibited a statistically significant increase under salt content S1 compared to S4 ($P < 0.05$). Inoculated alfalfa plants showed a decrease in MDA content of 14.41%, 15.62%, and 15.87% under salt treatments S1, S2, and S3, respectively, in comparison to non-inoculated alfalfa plants (Fig. 2a). In alfalfa leaves, the activity of the superoxide dismutase (SOD) enzyme was found to be influenced by both the salt concentration and the presence of *Bacillus tropicus* inoculation. Specifically, SOD enzyme activity exhibited a consistent decrease as salt content increased, with excessive salt concentrations leading to irreversible damage to plant cells and a subsequent decline in SOD enzyme activity (Fig. 2b). In the absence of bacterial inoculation, the peroxidase (POD) enzyme activity in alfalfa leaves showed a decreasing trend as the salt content increased (Fig. 2c). Following inoculation, the POD enzyme activity in alfalfa leaves showed an overall upward trend, reaching its maximum value (4969.15 $U \cdot g^{-1}$) under the BS4 treatment, with a significant difference compared to other treatments ($P < 0.05$). In summary, inoculation increased the activity of POD enzyme in alfalfa. Regardless of inoculation with *Bacillus tropicus* strain YJ33 strain, the CAT enzyme activity in alfalfa leaves showed an increasing trend with increasing salt content. However, inoculation significantly enhanced CAT enzyme activity in alfalfa leaves (Fig. 2d).

Following inoculation, a notable variance in phosphorus levels was observed across varying salt concentrations ($P < 0.05$). In contrast to the control group without inoculation, the phosphorus content in plants exhibited an upward trend with escalating salt concentrations, peaking under the BS4 treatment (4.63 g kg^{-1}) ($P < 0.05$). Additionally, within the same salt treatment, the phosphorus content in inoculated alfalfa surpassed that of non-inoculated alfalfa (Fig. 2e). Under the same salt content treatment, the crude protein content of alfalfa after inoculation was significantly higher than that without inoculation ($P < 0.05$), and

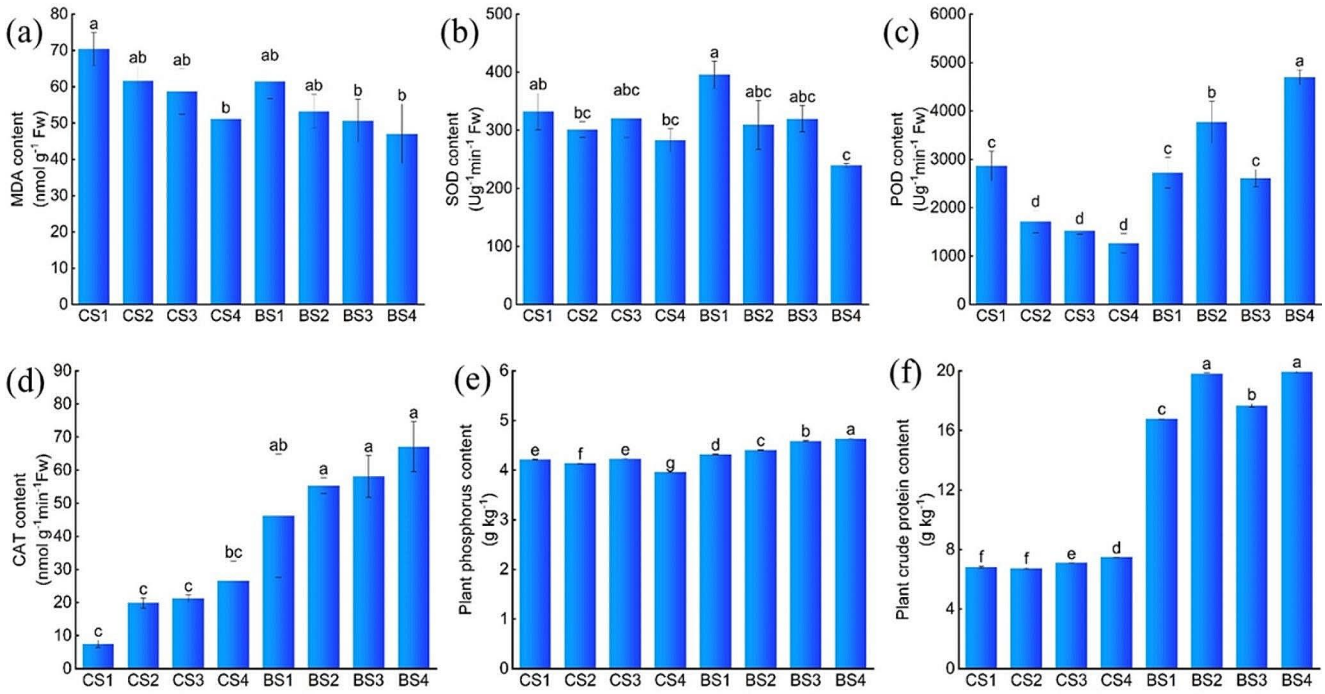

Fig. 2 The effects of inoculation and non-inoculation on enzyme activity and nitrogen and phosphorus of alfalfa under different salt concentrations. (**a**) Malondialdehyde (MDA) content, (**b**) superoxide dismutase (SOD) activity, (**c**) peroxidase (POD) activity, (**d**) catalase (CAT) activity, (**e**) phosphorus content, and (**f**) crude protein content. Values with different letters are significantly different at $p < 0.05$ by variance analysis

inoculation increased the crude protein content of alfalfa under salt stress (Fig. 2f).

In terms of soil properties, under the same salt content treatment, the soil carbon content under CS1 and BS1 treatments was highest ($P < 0.05$) under non-inoculation and inoculation treatments, respectively (Fig. 3a). As the salt content increased, the soil nitrogen content showed a downward trend. Under the same salt content conditions, the soil nitrogen content after inoculation treatment was 11.43%, 10.68%, 12.12%, and 6.80% higher than that under the non-inoculation treatment (Fig. 3b). Under the same salt content conditions, the soil carbon nitrogen ratio after inoculation was higher than that in the non-inoculation treatment (Fig. 3c), with the highest in BS1 treatment and the lowest in CS4 treatment ($P < 0.05$). The soil nitrate nitrogen content showed an upward trend with the increase in salt content when not inoculated with bacteria (Fig. 3d) and reached the maximum value (0.30 mg kg^{-1}) in BS4 treatment ($P < 0.05$). Under the same salt content conditions, the soil nitrate–nitrogen content after inoculation treatment was 1.25%, 25%,8%, and 15.38% higher than that after the non-inoculation treatment. Throughout the inoculation treatment, there was a noticeable rise in soil ammonium nitrogen content as salt concentration levels increased (Fig. 3e). The highest soil ammonium nitrogen content was observed in the BS4 treatment (3.30 mg kg^{-1}; $P < 0.05$), while the lowest soil ammonium nitrogen content was observed in the CS2 treatment (0.08 mg kg^{-1}). The ammonium nitrogen content in the soil treated with inoculation was significantly higher than that of the non-inoculation treatment ($P < 0.05$). When not treated with bacteria, the soil's available phosphorus content was the highest under S1 treatment (Fig. 3f). In the bacterial inoculation treatment, the soil available phosphorus content showed an overall increasing trend with the increase in salt concentration, reaching its maximum value ($P < 0.05$) in the BS4 treatment, and the soil available phosphorus content was the lowest in the BS1 treatment. At the same salt content, except for the S1 salt concentration treatment, the soil available phosphorus content of the inoculated treatment was significantly higher than that of the non-inoculated treatment under other salt concentrations.

3.2 Effect of Inoculation on Soil Microbial Communities

Following inoculation with *Bacillus tropicus* strain YJ33, the Sobs, Shannon, ACE, and Chao indices at the OTU level of soil bacteria decreased compared to the non-inoculated treatment (Table S2), indicating that inoculation had a certain impact on the structure of the soil microbial community. The diversity of microorganisms has decreased. The species coverage of all samples in the table was above 99%,

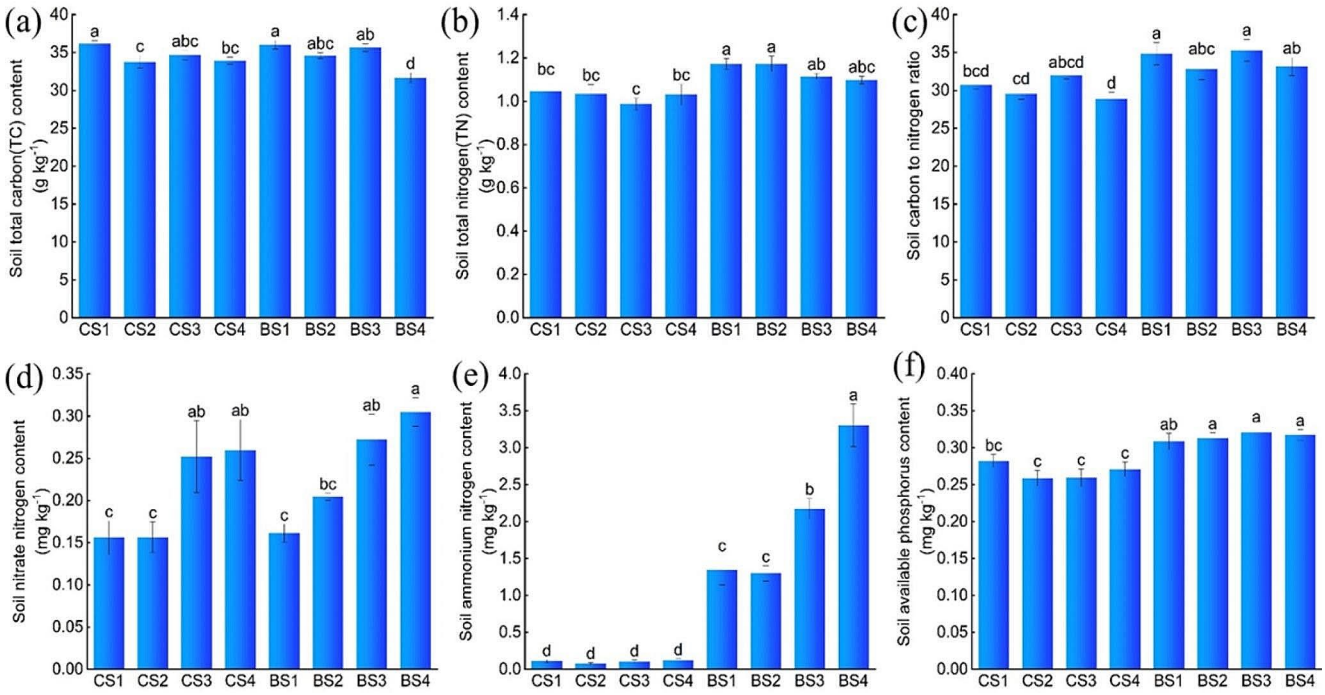

Fig. 3 The effects of inoculation and non-inoculation on soil properties under different salt concentrations. (**a**) total carbon content, (**b**) total nitrogen content, (**c**) carbon nitrogen ratio, (**d**) nitrate nitrogen content, (**e**) ammonium nitrogen content, and (**f**) available phosphorus content. Values with different letters are significantly different at $p < 0.05$ by variance analysis

indicating that the sequencing results reflected the authenticity of microorganisms in the soil samples. Under S1, S2, S3, and S4 salt treatments, the Shannon index of family level was significantly reduced ($P < 0.001$) compared to the non-inoculated treatment (Fig. 4a), indicating a change in bacterial community diversity after inoculation. The Chao index of family level was significantly reduced only at S1 salinity in the non-inoculated treatment, and there was no significant change compared to the control after the other inoculated treatments (Fig. 4b). The composition of the microbial community exhibits variability in response to environmental fluctuations. As depicted in Fig. S4, following inoculation, *Bacillus tropicus* predominated among the bacterial species, while the population of *Rhizobium*, or rhizobia, displayed an upward trajectory, mirroring the heightened presence of rhizobia in alfalfa roots. Furthermore, post-inoculation, the abundance of *Glutamicibacter arilatitensis* in the soil exhibited an increase. To compare the similarities between the groups, PCoA based on the Bray–Curtis distance matrix was performed at the family level (Fig. S5). The analysis of inter-group differences in bacterial β diversity further demonstrated that there was a significant difference in community structure composition between the non-inoculated and inoculated treatments ($P < 0.001$), and there was no significant difference in species composition between the non-inoculated and inoculated treatments ($P < 0.001$).

In all bacterial communities, 24 phyla, 297 families, and 641 genera were detected with a clear classification status. Among all categories, the relatively abundant microbial communities were Proteobacteria, Firmicutes, Bacteroidota, and Actinobacteriota (Fig. 5). Compared with the non-inoculated treatment (18.55%), the abundance of Firmicutes significantly increased after inoculation with *Bacillus tropicus* (48.37%), while the abundance of Proteobacteria (26.88%) decreased compared to the non-inoculated treatment (46.06%). The abundance of Actinobacteriota (4.78%) decreased compared to the non-inoculated (13.49%).

Following this, an analysis was conducted on the influence of plant environmental factors on bacterial communities at the family level using Redundancy Analysis (RDA), revealing that the combined variables accounted for 83.87% of the explanatory power on bacterial communities (Fig. 6a). Following inoculation, the biomass, crude protein content, phosphorus content, CAT content, and POD content of alfalfa were highly correlated with the soil bacterial community. Through RDA analysis of the impact of soil environmental factors on bacterial communities at the family level, the explanatory power of variable combinations on bacterial communities was 68.92% (Fig. 6b). Following inoculation, there was a strong correlation between soil ammonium nitrogen and available phosphorus with the soil bacterial community.

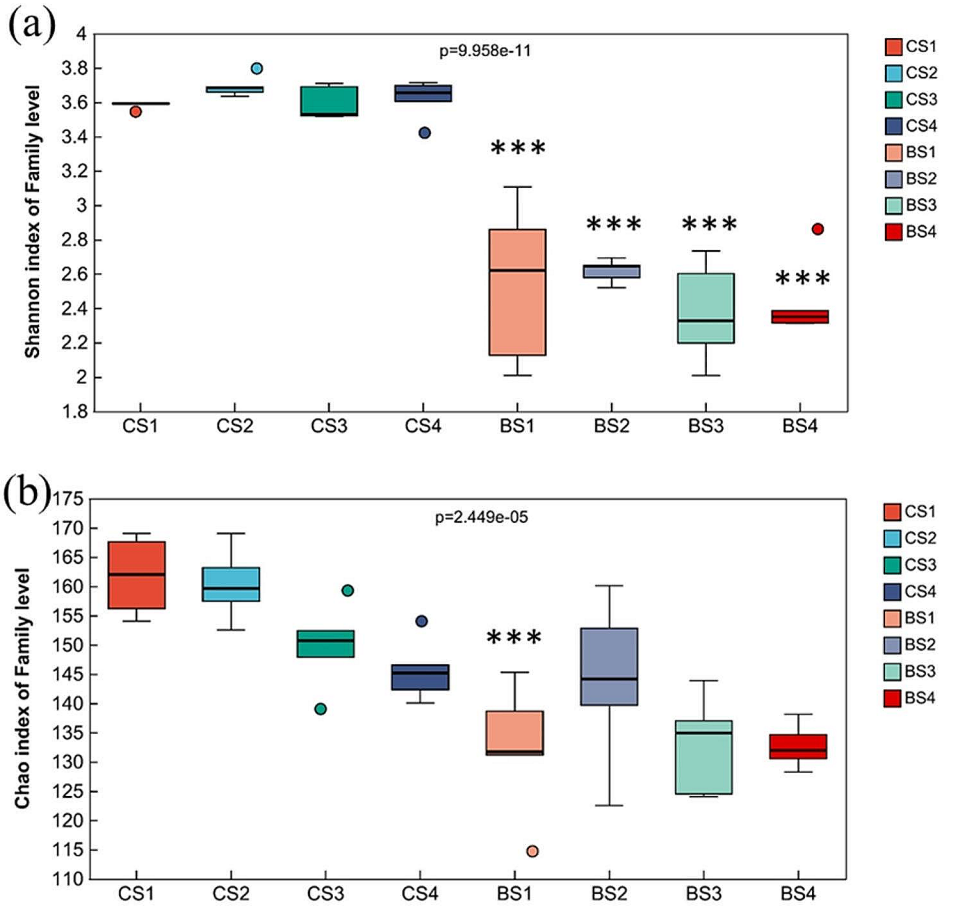

Fig. 4 The One-way analysis of variance (ANOVA) was conducted to compare the Shannon and Chao indices at the family level between inoculation and non- inoculation treatments across various salt concentrations. * represents significant differences; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

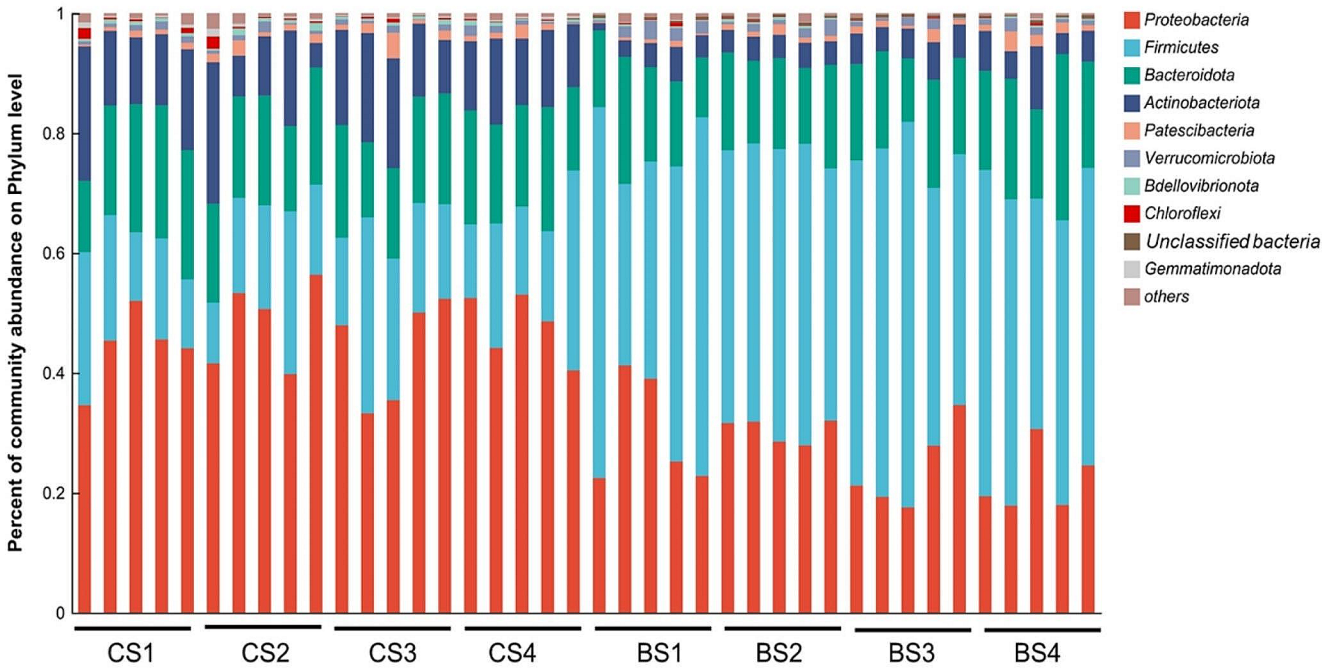

Fig. 5 The top 10 most abundant bacteria at the phylum level exhibited dominance in both inoculated and non-inoculated treatments under different salt concentrations

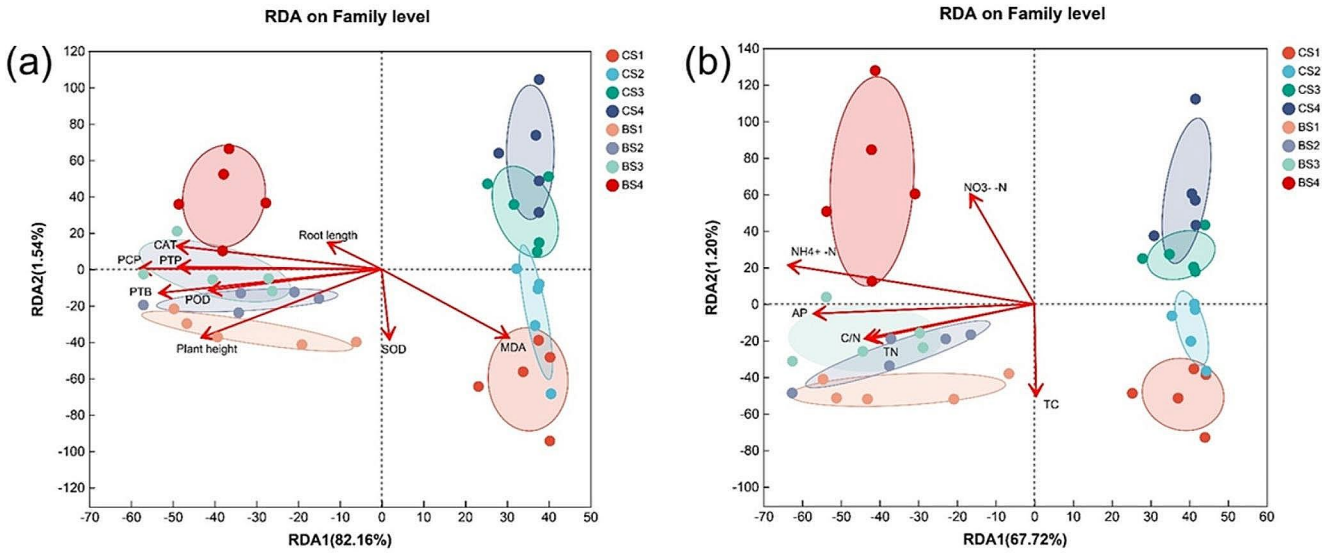

Fig. 6 The RDA analysis of plant environmental factors (**a**) and soil environmental factors (**b**) of bacterial communities at the family level between inoculation and non-inoculation treatments across various salt concentrations. MDA: malondialdehyde; SOD: superoxide dismutase; POD: peroxidase; CAT: catalase; PTP: plant total phosphorus; PCP: plant crude protein; PTB: plant total biomass

Spearman correlation analysis showed that the phosphorus and carbon content of alfalfa were significantly positively correlated with Firmicutes ($P<0.001$), negatively correlated with Proteobacteria and Actinobacteriota ($P<0.001$), and weakly negatively correlated with Bacteroidota ($P<0.05$). The crude protein content, total biomass, and plant height of alfalfa were significantly positively correlated with Firmicutes ($P<0.001$) and negatively correlated with Proteobacteria and Actinobacteriota ($P<0.001$). MDA was significantly positively correlated with Proteobacteria ($P<0.01$), weakly positively correlated with Actinobacteriota ($P<0.05$), and significantly negatively correlated with Firmicutes ($P<0.01$). POD and CAT showed a highly significant positive correlation with Firmicutes ($P<0.001$) and a highly significant negative correlation with Proteobacteria and Actinobacteriota ($P<0.001$). The soil nitrogen, ammonium nitrogen, and available phosphorus content showed a significant negative correlation with Proteobacteria ($P<0.01$), a highly significant negative correlation with Actinobacteriota ($P<0.001$), and a highly significant positive correlation with Firmicutes ($P<0.001$; Fig. 7).

3.3 Effects of Inoculated Strains on Soil Properties and Alfalfa Physiology and Growth

The structural equation model (SEM) was used to analyze the effects of inoculation on the soil nutrients and physiology and growth of alfalfa (Fig. 8). The model had a high fitting rate ($\lambda^2=0.401$, $P=0.589$, GFI = 0.992, RMSEA = 0.000), indicating a good model fit. The model explained 65.9% of soil ammonium nitrogen changes, 52.5% of soil available phosphorus changes, 72.2% of plant phosphorus changes, 93.1% of plant nitrogen changes, and 89.9% of plant biomass changes. The abundance of strain YJ33 significantly affected soil ammonium nitrogen ($P<0.001$), soil available phosphorus ($P<0.001$), soil ammonium nitrogen and plant phosphorus ($P<0.001$), and plant crude protein content ($P<0.001$). Strain YJ33 significantly affected plant crude protein content ($P<0.001$), and plant crude protein content significantly affected plant biomass ($P<0.001$). Strain YJ33 significantly affected plant biomass ($P<0.001$).

4 Discussion

Rhizosphere microorganisms are important components of the soil microenvironment that not only promote plant growth but also enhance plant resistance to various environmental stressors (Shah et al. 2017; Shrivastava and Kumar 2015; Spanu and Panstruga 2017). Salinity can affect the composition, interaction, and assembly of microbial communities (Li et al. 2021a). Research has shown that microorganisms can alleviate the damage suffered by plants under high salinity, drought, disease, and metal pollution (Liu et al. 2017; Wang et al. 2016; Wu et al. 2018). This study conducted a pot experiment on a bacterial strain, *Bacillus tropicus* strain YJ33, which was isolated by the research group. YJ33 remained active at a salt concentration of 1000 mmol/L. YJ33 affected plant leaf enzyme activity, plant nutrient content, soil nutrient content, and the soil microbial community.

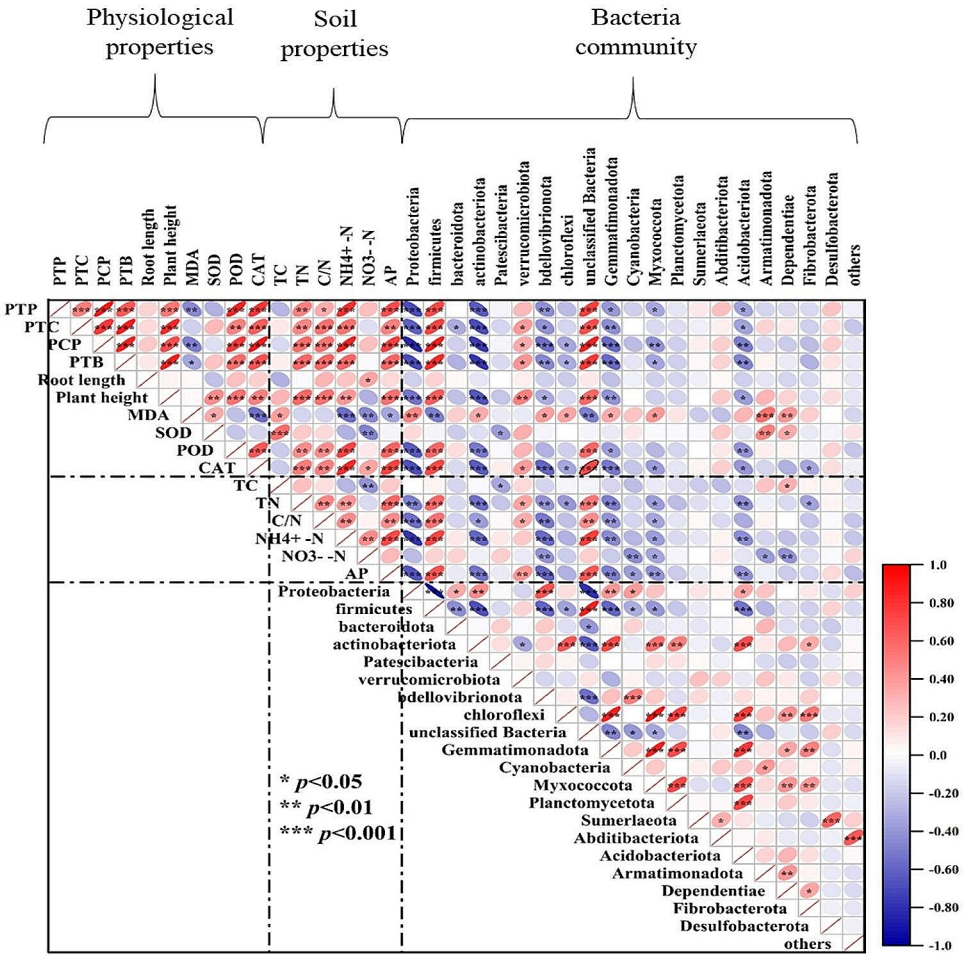

Fig. 7 Spearman correlation analysis of physiological properties and soil properties and bacteria community of soil bacterial phyla between inoculation and non-inoculation treatments across various salt concentrations. *, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. PTP: plant total phosphorus; PTC: plant total carbon; PCP: plant crude protein; PTB: plant total biomass; MDA: malondialdehyde; SOD: superoxide dismutase; POD: peroxidase; CAT: catalase; TC: soil total carbon; TN: soil total nitrogen; C/N: soil total carbon and nitrogen ratio; NO_3^--N: nitrate nitrogen; NH_4^+-N: ammonium nitrogen; AP: soil available phosphorus

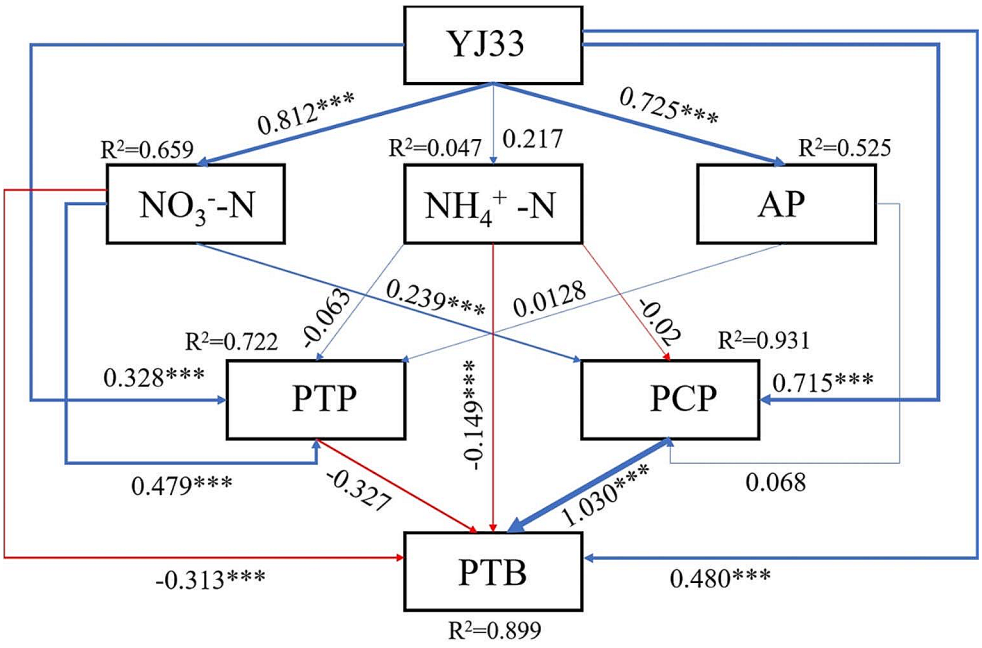

Fig. 8 Structural equation model (SEM) among the inoculated strains (YJ33), soil properties, alfalfa physiology, and growth. The values on the SEM line represent the path coefficient, and * represents significant differences (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$). The blue line represents the positive path coefficient; the red line represents the negative path coefficient; and the width of the arrow indicates the size of the standard path coefficient. NO_3^--N: soil nitrate nitrogen; NH_4^+-N: soil ammonium nitrogen; AP: soil available phosphorus; PTP: plant total phosphorus; PCP: plant crude protein; PTB: plant total biomass

Following inoculation with strain YJ33, there was a notable increase in the population of rhizobia in the rhizosphere of alfalfa compared to the non-inoculated control. However, as salt concentration levels rose, this symbiotic relationship began to deteriorate, even at a concentration of 300 mmol/L, irrespective of the presence of root nodules on the inoculated plants, due to the inhibitory effects of salt stress on the metabolic activities of rhizobia (Ahmad et al. 2013). In this study, the alfalfa growth in the non-inoculated group exhibited characteristics indicative of stunted growth, including diminished plant height, yellowing leaves, and a decreased leaf count. This may be attributed to the plant's adoption of a conservative growth strategy in response to environmental stressors, such as limited water or nutrient availability, resulting in reduced overall plant height. Alfalfa was planted in nutrient soil in this experiment. The nutrients in the control treatment may not have been sufficient to supply plant growth, leading to plant nutrient deficiency, yellowing, or even the falling of leaves. However, after inoculation, this situation was greatly alleviated. Li et al. (2021a) found that the chlorophyll content of WT plants significantly decreased by 48.7% and 75% after alkali stress and high salt stress, respectively. After salt stress, the chlorophyll content of transgenic plants significantly decreased ($P < 0.05$), while there was no change in chlorophyll content after alkali stress, which is consistent with the results of this experiment.

The impact of PGPR on plant growth can be observed through various indicators of biomass, such as plant height, root length, and fresh weight, which provide insight into the plants' growth status and their capacity to assimilate and utilize nutrients. Wei et al. (2022) used *Cynodon dactylon* roots as the research material and treated them with and without bacteria at a 250 mmol salt concentration. They found that the lawn quality of *Cynodon dactylon* roots treated with bacteria was significantly improved, and the fresh weight of the roots and buds increased by 84.8% and 47.4%, respectively. Research findings indicate that PGPR, including Pseudomonas, Enterobacter, and Bacillus, have the potential to enhance plant growth, increase stress tolerance in plants exposed to drought, saline, and alkaline conditions, and bolster plant immunity against diseases (Flor-Peregrín et al. 2014). From a phenotypic standpoint, the inoculation of alfalfa with YJ33 has been shown to enhance its growth under high salt concentration stress conditions, thereby increasing its resilience. The results of this study indicate that while the aboveground biomass of alfalfa generally decreased with increasing salt concentration, inoculation with YJ33 significantly improved both aboveground biomass and plant height, as demonstrated in Fig. 1A and B. These findings are in line with previous research by Wei et al. (2022). There was no significant difference in alfalfa root length among the treatments, which may be due to the low height of the flowerpot selected in the early stage of this experiment, which restricted alfalfa root growth (Fig. 1c). When plants are stressed, their free radical content increases, which causes oxidative damage to plant cells. Free radicals undergo peroxidation, and the final product is MDA (Chen et al. 2009). MDA damages the structure of the plant cell membrane. The lower the MDA content in plant leaves showed the stronger stress resistance and the less damaged (Abd El-Baki and Mostafa 2014). Several studies have indicated that MDA content can serve as a physiological indicator of both low and high salt tolerance under salt stress conditions, with a negative correlation observed between MDA content and seedling salt tolerance (Zhang et al. 2019). According to the analysis of the MDA content under the same salt content conditions, inoculation reduced the response of alfalfa to salt stress, relatively reduced the MDA content, and helped plants slow the toxic effects of MDA. Yasin et al. (2018) found that, after inoculation with *Bacillus fortis* strain SSB21, the proline content significantly increased and the MDA content significantly decreased, indicating that the stress on plants was alleviated, which is consistent with the results of this experiment. In this study, when alfalfa was under salt stress, the SOD content in its leaves decreased with the increase in salt content, but after inoculation with strain YJ33, the SOD content in plant leaves increased to varying degrees, indicating that strain YJ33 improved the activity of protective enzymes in alfalfa leaves to maintain redox homeostasis in cells. In addition to the obvious increase in SOD content, the POD and CAT content also increased with the increase in salt concentration, which was opposite to the increase in SOD. The maximum value of POD and CAT appeared at a salt concentration of 300 mmol/L, indicating that under the stress of higher salt concentration, strain YJ33 could also help plants activate the antioxidant system to resist salt damage. The levels of peroxidase (POD) and catalase (CAT) content exhibited a notable increase following inoculation, suggesting that the introduction of strain YJ33 substantially enhanced the salt stress tolerance of alfalfa (Koranda et al. 2011). However, the mechanisms underlying the changes in various physiological indicators under high salt treatment still need to be further studied. In this study, it was observed that the phosphorus content of alfalfa decreased with increasing salt concentration in the absence of inoculation. However, significant increases in phosphorus content were noted after inoculation with strain YJ33, showing a negative correlation with salt concentration. It is hypothesized that strain YJ33 may have enhanced the growth conditions of root nodules, leading to increased levels of ammonium nitrogen and nitrate nitrogen, thereby promoting phosphorus absorption by alfalfa in the soil. These findings align with previous

research conducted by (Zaefarian Faezeh et al. 2011). Salt stress inhibits the uptake of soil nutrients by alfalfa, resulting in leaf yellowing or abscission. Additionally, osmotic stress caused by salt can lead to protein degradation. The results of this experiment demonstrate that the crude protein content of inoculated alfalfa was significantly higher than that of non-inoculated alfalfa, consistent with previous findings (Vessey and Heisinger 2001). Rhizosphere PGPR increases the availability of plant rhizosphere nutrients through a series of complex mechanisms, produces ferritin, and assists other major symbiotic relationships to enable better accumulation of crude protein in alfalfa and improve its quality. Following the inoculation of alfalfa, there was a notable increase in nitrogen and phosphorus content, indicating that the addition of microorganisms can enhance the synthesis and metabolism of these nutrients through intricate mechanisms. This enhancement significantly enhances the production performance of alfalfa, effectively improving its quality and achieving the dual objectives of high yield and quality. Soil salinization has the potential to induce nutrient depletion in soil, and the uptake of soil nutrients by plants is intricately linked to elemental composition. Alterations in microbial communities due to changes in salt levels may impact the carbon cycle within soil, as fungi have a preference for decomposing complex organic compounds like cellulose and lignin, whereas bacteria tend to utilize more readily available organic carbon sources (Paterson et al. 2008). Geml et al. (2014) also found that the soil bacterial community is closely related to soil carbon and nitrogen content. However, our research conducted revealed a negative correlation between salt concentration and soil carbon content, with an observed increase in plant carbon content in response to strain YJ33. This phenomenon may be attributed to the enhancement of carbon source absorption efficiency by alfalfa under stress conditions, thereby facilitating the accumulation of element reserves for subsequent growth. Simultaneously, the experiment demonstrated a notable increase in soil nitrogen content following inoculation, aligning with previous research by Kheirfam et al. (2017) which indicates that the introduction of microorganisms can enhance carbon and organic storage, thereby enhancing soil chemical characteristics. Wang et al. (2022a) showed that the addition of biochar and manure increased the content of soil nitrate nitrogen, ammonium nitrogen, and available phosphorus. Similarly, the content of nitrate nitrogen, ammonium nitrogen, and available phosphorus increased significantly after inoculation in this experiment, especially when the salt concentration was 225 or 300 mmol/L, the content of the above three indicators was significantly higher than that in the low salt concentration treatment, the reason may be that the strain can activate nitrogen and phosphorus elements in the soil under high salt conditions, providing nutrients for plant survival (Shoda and Ishikawa 2014). The study observed a notable increase in available phosphorus in the soil, potentially attributed to the reduction of metabolic elements by YJ33 under high salt concentrations. This phenomenon led to a significant rise in soil nutrient levels. Throughout the growth cycle of YJ33, the microorganism sequesters phosphorus, which is subsequently released into the soil as inorganic phosphorus upon its demise, serving as a source of phosphorus for plant growth (Turner et al. 2003). Harishankar et al. (2013) found that both *Lactobacillus lactis*can and *L. fermentum* produce organic phosphorus phosphatase (OPP), which can hydrolyze organophosphorus and release phosphate.

Soil microorganisms play an important role in maintaining soil ecosystem functions, including organic matter decomposition, nutrient cycling, bioremediation, soil organic matter stabilization, and soil aggregate formation (Dangi et al. 2018). Nitrogen-fixing bacteria utilize the enzyme nitrogenase to catalyze the conversion of atmospheric nitrogen into ammonium, a process commonly referred to as biological nitrogen fixation. The fixed nitrogen is subsequently incorporated into amino acids rather than being released into the surrounding environment. This assimilation of ammonium by bacteria typically involves the synthesis of glutamic acid and glutamine from ammonium and 2-oxoglutaric acid (Li et al. 2022). Glutamic acid bacteria can produce glutamic acid, which is one of the basic amino acids for nitrogen metabolism in biological organisms and is of great significance. Following inoculation, soil microorganisms facilitate the conversion of nitrogen and phosphorus elements, which are typically inaccessible to plants, into forms that are readily absorbable and utilizable by plants. This process ultimately results in an increase in the nitrogen and phosphorus content within plants. Firmicutes and Proteobacteria play an important role in nitrogen transformation and release (Wang et al. 2023; Yang et al. 2019c). Following inoculation with strain YJ33, there was a notable increase in the abundance of Firmicutes, suggesting that the strain has the potential to enhance nitrogen availability. The abundance of Proteobacteria decreased after inoculation, and microbial diversity was affected by biological and abiotic factors (Singh et al. 2009). The phosphorus content, carbon content, crude protein content, total biomass, plant height, POD, and CAT of alfalfa were significantly positively correlated with Firmicutes. The soil nitrogen content, ammonium nitrogen, and available phosphorus were significantly positively correlated with Firmicutes, indicating that the inoculation of strain YJ33 improved the soil environment by affecting the abundance of some phyla related to nitrogen transformation and significantly increasing the soil nutrient content. Promoting carbon, nitrogen, and phosphorus anabolism significantly improved the production performance of alfalfa,

which is consistent with Ku et al. (2021). Inoculation did not significantly enhance the overall diversity of microorganisms in the soil; however, it did alter the abundance of specific categories, potentially as a result of the activation of particular microorganisms post-inoculation, as well as the suppression of certain bacterial growth, leading to a reduction in microbial diversity (Peng et al. 2017).

5 Conclusion

Our research has identified a strain of *Bacillus tropicus* (YJ33), isolated from the rhizosphere of *Suaeda salsa*, that has the potential to enhance the physiological characteristics of alfalfa through alterations in soil microorganisms and physicochemical properties, ultimately leading to improved growth and salt stress tolerance. Based on the structural equation model, the presence of strain YJ33 in the soil resulted in notable increases in ammonium nitrogen and available phosphorus content, along with elevated the biomass and nutritional quality in alfalfa. Furthermore, the nitrogen content in alfalfa was found to have a significant effect on its biomass. These results indicate that YJ33 is directly related to soil characteristics and alfalfa physiological characteristics. Nevertheless, further investigation is required to elucidate the specific mechanisms underlying its salt tolerance and growth-promoting effects.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42729-024-01828-4.

Acknowledgements This study was supported by the National Natural Science Foundation of China (32101434), the National Key Research and Development Program of China (2022YFD1300802), the Agricultural Research System of China (CARS-34), the Qingdao Agricultural University Doctoral Start-Up Fund (1120025), and the "Youth Innovation Team Plan" of Universities in Shandong Province (2022KJ166).

Declarations

Competing Interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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