

Bacillus subtilis HG-15, a Halotolerant Rhizoplane Bacterium, Promotes Growth and Salinity Tolerance in Wheat (*Triticum aestivum*)

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Abstract

Background : Certain plant growth-promoting bacteria (PGPB) reduce salt stress damage in plants. *Bacillus subtilis* HG-15 is a halotolerant bacterium (able to withstand NaCl concentrations as high as 30%) isolated from the wheat rhizosphere in the Yellow River delta. A qualitative and quantitative investigation of the plant growth-promoting characteristics of this strain confirmed nitrogen fixation, potassium dissolution, and ammonia, plant hormone, 1-aminocyclopropane-1-carboxylic acid deaminase, and proline production abilities. *B. subtilis* HG-15 colonization of wheat roots, stems, and leaves was examined via scanning electron microscopy, rep-PCR, and double antibiotic screening.

Results : Compared with a no *B. subtilis* HG-15 treatment control, in rhizosphere soil inoculated with the HG-15 strain, the pH (1.08–2.69%), electrical conductivity (3.17–11.48%), and Na⁺ (12.98–15.55%) concentrations significantly decreased ($p < 0.05$). Inoculation with the HG-15 strain increased the total N, available N, organic matter, K⁺, Ca²⁺, and Mg²⁺ concentrations in the rhizosphere soil of wheat. Under non-salt stress (0.15% NaCl), low-salt stress (0.25% NaCl), and high-salt stress (0.35% NaCl) conditions, respectively, this strain also significantly increased ($p < 0.05$) the dry weight (17.76%, 24.46%, 9.31%), fresh weight (12.80%, 20.48%, 7.43%), plant height (7.79%, 5.86%, 13.13%), root length (10.28%, 17.87%, 48.95%), and other wheat parameters. Through redundancy analysis and Pearson correlation analyses, photosynthesis, biomass accumulation, and osmotic regulation by the wheat plants showed a significant negative correlation with pH, EC, and Na⁺ concentrations in rhizosphere soil.

Conclusions : Our results indicated that *B. subtilis* HG-15 can effectively improve the growth of wheat and elicit induced systemic tolerance in these plants, thus, showing its potential as a microbial inoculant that can protect wheat in salt stress conditions. Furthermore, we determined that the rhizosphere of saline-alkali land plants is an important reservoir for salt-tolerant PGPB.

Background

Soil salinization is a serious abiotic stress factor that affects crop production; approximately 20% of the world's cultivated land is currently under threat from varying degrees of salinization. As fertile land available for cultivation decreases, soil salinization intensifies, owing to irrational irrigation and fertilization [1]. Salt stress reduces the yield of important food crops such as wheat, corn, rice, and barley by approximately 70% [2]. The Yellow River delta is the fastest salinizing coastal ecosystem in the world. This area is abundant in light, relatively cool, highly saline in soil, and prone to drought, waterlogging, and alkalization. Wheat is one of the most important cereals in the world and the main food crop grown in the Yellow River delta. As soil salinity increases, wheat growth and yield are threatened [3]. Reducing the damage to wheat from long-term exposure to high salt concentrations and promoting the growth of wheat in low-salt concentration soils is a priority, because there is a significant amount of coastal saline-alkali and secondary saline-alkali soil in this area.

Salt stress can destroy plants through secondary processes such as the promotion of ion stress, osmotic stress, and oxidative stress [4]. Na⁺ and Cl⁻ are the predominant salt ions in saline soil; they are toxic to plants and also inhibit the absorption of other nutrient ions, which results in nutrient deficiency [5]. Soil salinization reduces the energetic state of rhizosphere soil water, causing the osmotic pressure of the soil solution to exceed the normal osmotic pressure in plant cells, which results in osmotic stress [6]. This leads to changes in the length, density, and structure of plant roots, reducing the uptake of soil water and nutrients, which causes plants to enter a state of physiological drought and even die [7]. Furthermore, oxidative stress causes damages to the cell membranes and photosynthetic system [8].

Upadhyay et al. [9] found that plant growth-promoting bacteria (PGPB) lose plant growth-promoting (PGP) traits with increased salinity in vitro. Thus, the use of halotolerant PGPB that are selected based on both high salt tolerance and efficiency in expressing PGP traits could significantly advance our ability to grow crops in environments with natural or induced salinity [1]. Similar to halophytes, salt-tolerant rhizobacteria have evolved strategies to adapt to high-salt environments. An important strategy is the ability to accumulate compatible osmolytes to maintain homeostasis in cells [10, 11]. These bacteria exhibit various stress-related properties that may protect plants at growth-inhibiting levels of salts. PGPB, together with plant roots and soil, form a special soil microenvironment termed the rhizosphere, which is considered the single most important biological factor for plant growth [12]. Although research related to the effects of PGPB on the rhizosphere are far from comprehensive, several studies have shown that the application of microbial agents may significantly reduce soil pH, decrease salinity, improve saline-alkali land management, and effectively increase the survival rate of plants grown in saline-alkali soil [13]. Antimicrobial substances and peroxidase (POD) may also be induced by PGPB to participate in plant defense responses and improve the resistance of plants to diseases and pests [14]. PGPB fix nitrogen (N), dissolve potassium and indole-3-acetic acid (IAA), promote iron carriers and 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD) activity, and stimulate

other factors that directly affect plant growth. PGPB also secrete extracellular polysaccharides (EPS), osmotic regulators, and various volatile organic compounds (VOCs) that alter the structure and morphology of roots and elicit induced systemic tolerance (IST) in plants [15]. Therefore, it is important to screen salt-tolerant PGPB for their degree of salt stress tolerance and ability to express PGP traits [1]. At the same time, the physiological and biochemical characteristics of these salt-tolerant bacteria protect plants and restore the plant-growth potential of saline soils [16]. In addition, endophytes not only show a colonization advantage but also show benefits to host plants by reducing stress damage and increasing root growth [17].

Few studies have screened PGPB with plant growth potential in the Yellow River delta area. In this study, a bacterial strain with high salt tolerance was isolated from the rhizosphere of wheat planted in saline-alkali soil. By testing PGP activity and the ability of the plants to colonize rhizosphere soil, we found that this strain had the potential to reduce salt tolerance and promote plant growth. The effects of salt-tolerant PGPB on the growth of wheat and chemical properties of the rhizosphere soil were examined. In addition, we determined the correlation between rhizosphere soil and plant and salt tolerance parameters. The purpose of this study was to understand the PGP characteristics of the PGPB strain and its effect on wheat growth in saline-alkali soil.

Results

Isolation, biochemical characterization, and identification of HG-15

Biochemical and physiological characteristics of HG-15 are shown in **Table 1**. The 16S rRNA sequences of HG-15 were analyzed at the molecular level, where they were found to be most closely matched (99%) with 16S rRNA of *Bacillus subtilis* AJ276351 (**Fig. 1**). The strain HG-15 sequence was deposited in the China General Microbial Culture Collection under accession number 15773. The HG-15 16S rRNA sequence was deposited in the NCBI database under accession number MN689681.

Salt tolerance and plant growth-promoting features

Among the isolated rhizosphere bacteria, the HG-15 strain showed the most pro-growth potential, and its PGP activity was examined. The test strain HG-15 had high salt tolerance and was inoculated into LB liquid medium at 30% NaCl concentration for 7 d. After the strain was mixed with high-salt liquid hydrolyzed fertilizer and solid water-soluble fertilizer for 2 years, the strain recovery rates reached 82.63% and 85.37%, respectively. 1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity was $14.816 \pm 0.965 \mu\text{mol}/(\text{mg}\cdot\text{h})$, whereas IAA production was $154.53 \pm 4.17 \mu\text{g}/\text{mL}$. ABA, GA₃, ZA, JA, and SA content determined via HPLC at 3–7 d was $0.609 \pm 0.026 \mu\text{g}/\text{mL}$, $0.103 \pm 0.005 \mu\text{g}/\text{mL}$, $0.638 \pm 0.014 \mu\text{g}/\text{mL}$, $0.430 \pm 0.016 \mu\text{g}/\text{mL}$, and $3.865 \pm 0.098 \text{ ng}/\text{mL}$, respectively. Other PGP characteristics were analyzed and are summarized in **Table 2**. The HG-15 strain inhibited *Fusarium oxysporum* (84.34%), *Fusarium pseudograminearum* (80.21%), *Rhizoctonia solani* (87.84%), *Fusarium graminearum* (79.54%), and *Botryosphaeria ribis* (65.70%), but it did not inhibit *Botryosphaeria dothidea* (**Table S1**) (**Fig. S2**).

NaCl stress effects of HG-15 on soil chemistry properties under NaCl stress conditions

To evaluate the effects of HG-15 on wheat rhizosphere soil, we measured changes in pH, EC, and nutrient and metal elements in wheat rhizosphere soil. We then determined the growth of wheat in different soil concentrations. The rhizosphere soil pH decreased by 2.69%, 1.91%, and 1.08% at 0.15%, 0.25%, and 0.35% NaCl salt concentration, respectively ($p < 0.05$). Inoculation with HG-15 did not produce a significant difference in pH between soil with 0.25% and 0.35% salt concentrations. However, the pH of soil with a 0.15% salt concentration decreased significantly (**Fig. 2A**). Compared with that of the uninoculated strains, the rhizosphere soil EC decreased by 3.17%, 11.48%, and 4.91% at 0.15%, 0.25%, and 0.35% NaCl salt concentrations, respectively ($p < 0.05$). The higher the salt concentration, the higher the EC value ($p < 0.05$). The rhizosphere soil organic matter increased by 19.23%, 16.08%, and 10.08% at 0.15%, 0.25%, and 0.35% NaCl salt concentration, respectively ($p < 0.05$).

Compared with that of the uninoculated strain, inoculation with the HG-15 strain did not significantly change the Olsen-P content in rhizosphere soil (**Fig. 2D**). In both the control group and the HG-15 group, the higher the salt concentration, the lower the total N content ($p < 0.05$). Under the same salinity, the total N content and available N in rhizosphere soil inoculated with HG-15 was higher than that in the control group ($p < 0.05$) (**Fig. 2E, F**). Sodium ions, however, showed the opposite trend. Compared with that of the uninoculated strain, inoculation with the HG-15 strain increased the potassium content in the soil with concentrations of 0.15% ($p < 0.05$, 13.97%) and 0.35% ($p < 0.05$, 20.24%), but did not significantly affect soil at a 0.25% salt concentration (**Fig. 2H**). Inoculation with the HG-15 strain significantly increased the content of Mg ions in rhizosphere soil ($p < 0.05$), but did not affect Ca²⁺ content. Similarly, Ca²⁺ and Mg²⁺ content at a 0.35% salt concentration was significantly lower than that at 0.15% and 0.25% salt concentrations (**Fig. 2I, J**).

Effects of HG-15 on plant growth under salt stress

Plant growth and photosynthesis levels under salt stress

To study the effects of HG-15 on wheat growth under stress caused by different salt concentrations, we tested the biomass, photosynthesis, chlorophyll content, and chlorophyll fluorescence between HG-15 inoculated and uninoculated wheat seedlings after 28 d of growth. In the case of salt stress, the biomass (**Fig. 3**), photosynthesis, chlorophyll content, and chlorophyll fluorescence levels (**Fig. 4**) of the uninoculated seedlings were inhibited to various degrees. In the control group, there was no significant difference in dry weight and fresh weight at 0.25% and 0.35% salt concentrations, but there was a significant difference after HG-15 inoculation. Compared with the uninoculated treatment, the dry weight (9.31–24.46%) and fresh weight (7.43–20.48%) of wheat inoculated with the HG-15 strain were significantly increased ($p < 0.05$). However, the plant height and root length were not significantly increased. In the control group, there was no significant difference in dry weight and fresh weight at 0.25% and 0.35% salt concentrations, but there was a significant difference after HG-15 inoculation. In contrast, there were significant differences in plant height and root length in the control group ($p < 0.05$), but no significant differences in the HG-15 inoculation group (**Fig. 3**).

In the control group, soil with different salt concentrations had significant effects on *gsw*, *E*, and Chl a of wheat ($p < 0.05$). After inoculation of the HG-15 strain, different salt concentrations had significant effects on wheat *A*, *gsw*, and Chl b ($p < 0.05$). The higher the salt concentration, the lower the photosynthetic activity of wheat. At a 0.15% salt concentration, *E* (29.96%) was significantly increased in wheat inoculated with the HG-15 strain ($p < 0.05$). At a 0.25% salt concentration, Chl a (20.24%) content in leaves of wheat inoculated with the HG-15 strain was significantly increased ($p < 0.05$). At a 0.35% salt concentration, *E* (43.65%), and Chl a (28.68%) content increased significantly ($p < 0.05$) in wheat inoculated with the HG-15 strain (**Fig. 4**).

Biochemical analysis of plants treated with NaCl

The effect of HG-15 on the salt tolerance of wheat under salt stress can be evaluated by assessing changes in important chemical components such as proline, total soluble sugar (TSS), total protein, malondialdehyde (MDA), peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT). After testing these components at different levels, we found that proline levels in uninoculated plants were increased under salt stress conditions of 0.25% and 0.35%, indicating that salt stress levels of wheat were reduced following inoculation with the HG-15 strain, with proline showing less accumulation in HG-15-inoculated plants (**Fig. 5A**). Salt stress also significantly reduced the TSS content in wheat. With increasing salt concentrations, the TSS content in wheat inoculated with the HG-15 strain was higher than that in uninoculated wheat, with increases of 29.31% (0.15%), followed by increases of 17.91% (0.25%) and 43.06% (0.35%) ($p < 0.05$) (**Fig. 5B**). Salt stress also reduced the total protein content in uninoculated wheat by 16.36% (0.15%), 5.86% (0.25%), and 31.77% (0.35%), respectively (**Fig. 5C**). Total protein content was higher in wheat inoculated with the HG-15 strain compared to that in uninoculated wheat. The highest total protein content was observed in HG-15-inoculated plants under 0.25% salt concentrations, which showed a 31.48% increase over the uninoculated control (**Fig. 5C**).

Lipid oxidation damage in wheat is reflected by increased MDA content; therefore, MDA levels in wheat were estimated in relation to increased salt concentrations. Results indicated that the higher the salt concentration, the higher the MDA content in uninoculated wheat ($p < 0.05$). Following inoculation with HG-15, the MDA content at salt concentrations of 0.15%, 0.25%, and 0.35% were 21.63%, 24.34%, and 17.10% lower than those in uninoculated wheat, respectively (**Fig. 5D**). However, inoculation of the HG-15 strain did not significantly affect the POD activity of wheat. Results indicated that the higher the salt concentration, the lower the POD content in wheat ($p < 0.05$) (**Fig. 5E**). Compared with plants treated with similar NaCl concentrations, inoculation with HG-15 did not significantly change the SOD activity in wheat. Results indicated that the higher the salt concentration, the higher the SOD content in wheat ($p < 0.05$) (**Fig. 5F**). The results of this study indicated that the CAT activity of wheat increased with an increase in salt concentration. Inoculation with bacteria increased CAT activity of plants under salt stress, with a maximum increase of 18.38%, which was observed at a concentration of 0.15% NaCl. This increase became significant at 11.87% with a salt concentration of 0.35% (**Fig. 5G**).

Root colonization assay

The colonization efficiency of HG-15 in wheat roots, stems, leaves, and rhizosphere soil was determined by plate counts (with plates containing rifampicin and spectinomycin), rep-PCR measurement, and scanning electron microscopy. Changes in the number of bacteria following 7, 14, 21, and 28 d of rhizosphere colonization at different salt concentrations are shown (**Table 3**). The results showed that bacterial strain colonization changed the most from day 14 to day 21, and higher soil salt concentrations were associated with greater bacterial decreases. The least number of changes in the bacterial strain colonization were observed between days 21–28, as reflected by the absence of a significant difference. At a concentration of 0.15%, the number of bacteria decreased by 19.24%, 86.62%, and 92.42%,

respectively; at 0.25%, the number decreased by 32.95%, 92.00%, and 94.00% every 7 d; and at 0.35%, the number decreased by 62.59%, 95.01%, and 96.60% every 7 d (**Table 3**). Gene fingerprints of strains recovered from the wheat roots, stems, and leaves, and the HG-15 strain were compared using rep-PCR and found to be consistent, indicating that HG-15 can colonize wheat roots, stems, and leaves. The bacterial colonies and spore morphology of the isolated strains were similar, and no other strains with rifampicin resistance were detected in the soil; thus, there was no stripe information of other strains in **Fig. S1**. Scanning electron microscopy also showed that HG-15 colonized wheat stems, leaves, and roots (**Fig. 6**). The surface of treated roots, stems, and leaves were logically colonized, but no obvious bacteria were found on the root, stem, or leaf surfaces of uninoculated control plants.

Relationship between rhizosphere soil and plant factors

RDA analysis showed 85.85% of variation in soil and plant parameters (**Fig. 7**). According to the RDA analysis, total N, organic matter, and available N and Mg^{2+} were strongly positively correlated with plant growth, whereas EC, Na, and pH were strongly negatively correlated with plant growth (**Fig. 7**). Combined with the data in **Table S2**, we found the following: Na can explain 76.6% variation in plant growth, and OM, EC, and Ca^{2+} can explain 4.6%, 3.2%, and 2.2% in plant growth, respectively ($p < 0.05$). The results indicated that Na^+ content has a significant negative correlation with plant parameters. The effects of OM, EC, and Ca^{2+} on plant parameters were positively correlated.

We performed a correlation analysis of soil parameters and plant parameters under the effect of the HG-15 strain. As shown in **Fig. 8**, changes in various soil factors can change plant proline, total sugar, total protein, MDA, POD, Chl a, Chl b, A, *gsw*, *E*, DW, FW, plant height, and root length ($p < 0.05$). Available N, pH, Olsen-P, Mg, and Na had significant effects on plant factors ($p < 0.01$). In addition, pH, EC, and Na showed a strong negative correlation with plant photosynthesis and biomass accumulation. We also found that these factors were positively correlated with proline, MDA, CAT, and SOD content.

The main effects and interactions of salinity and bacteria on the measured parameters are shown in **Table 4**. Soil parameters considered for salt and bacteria interactions are pH, EC, organic matter, and total N. The plant parameters considered for salt and bacteria interaction are dry weight, fresh weight, plant height, root length, *gsw*, Chl a, proline, TSS, and total protein.

Discussion

The microorganisms used in soil-plant systems enhance crop viability, yield, quality, and tolerance to abiotic stress, and are therefore important agricultural biostimulants [18]. However, as several studies have shown, many microbial products are not always effective in the field [19]. Under salt stress, many strains also lose their PGP characteristics [1]. Etesami and Beattie 2017 [15] found that salt-tolerant endophyte have more stable salt-tolerant and PGP activity, which indicated that halophytes and saline-alkali environments are important resource banks for the isolation and screening of salt-tolerant strains. The range of PGPBs with multiple PGP traits that exist in the rhizosphere of halophytic plants is a valuable resource for improving crop tolerance to salinity and promoting saline soil-based agriculture in the future. Furthermore, studies on the use of PGPB to reduce crop salt stress damage are increasing. We emphasize that plant root surface is an important plant tissue for material exchange between plants and the external environment and direct contact with microorganisms. There are important microorganisms stored on the rhizoplane of plants in a saline alkali environment. These strains may have outstanding salt tolerance, PGP activity, and colonization ability in rhizosphere soil and plant interiors.

PGPB can synthesize the enzyme ACCD, which converts the plant ethylene precursor ACC to ammonia and α -ketobutyrate [15], thus reducing the accumulation of ethylene in the plant and avoiding ethylene-mediated growth inhibition in response to abiotic stresses such as salinity [20]. Furthermore, a variety of endophytic bacteria were found to have ACCD activity [21]. By comparing the ACCD activity of isolated strains in various habitats (including different tissues of plants), it was found that endophytic bacteria had higher ACCD activity than PGPB isolated from rhizosphere soil, non-rhizosphere soil, and other habitats [22]. ACCD activity has also become one of the most important screening criteria for salt tolerant PGPB. The results of this study showed that the isolated salt-tolerant bacteria HG-15 significantly increased the dry weight and fresh weight of wheat, and the root length of wheat was significantly increased by the inoculation of HG-15 under 0.35% salt concentration stress ($P < 0.05$) (**Fig. 3**). We speculate that this is closely related to the production of ACCD by the strain.

Research by Dunlap and Binzel [23] showed that salt stress can reduce endogenous IAA levels. At the same time, Zahir et al. [24] found that plants can also respond to exogenous plant hormones, which can alleviate the adverse effects of salinity. IAA production is a relatively common trait of most salt-tolerant PGPB [25], and IAA producing PGPB can increase the fitness of plants grown in salt-affected soils [26]. In this study, the HG-15 strain has the ability to produce IAA, which is important to help maintain leaf growth and reduce plant

productivity restrictions caused by saline-alkali stress [27]. PGPBs may improve crop salt tolerance by altering hormonal root shoot signaling [28]. Inoculating GA-producing PGPB can affect endogenous GA levels in plants [29], and help regulate plant leaf and root meristem size, cell division and elongation, hypocotyl and stem growth [30, 31]. Increased growth of drought-stressed lettuce plants inoculated with a ZA-producing *B. subtilis* strain suggested modulation of root-to-shoot ZA signaling [32]. ABA also plays a crucial role in plant-PGPB interactions [33]. For example, in a study, following the inoculation of plants with ABA-producing strains such as *B. licheniformis* Rt4M10, *P. fluorescens* Rt6M10, and *A. brasilense* Sp 245, the internal ABA content increased and inoculated plants became more resistant to drought compared to un-inoculated plants [34, 35]. The resulting changes in ABA levels may mitigate the plant's sensitivity to water scarcity. This study confirmed the promoting effect of HG-15 strain with multiple PGP characteristics (Table 2) on wheat plant growth under salt stress. Inoculation of this bacterium also regulates nutrient enrichment and Na^+/K^+ ratio in the rhizosphere soil of plants, thereby protecting plants from salt damage. Although there is evidence that PGPB improves plant salt tolerance by changing the state of endogenous hormones, how PGPB affects this process is unknown. We speculate that the hormones produced by PGPB in vitro and the hormones produced in plants can be recognized and utilized by plants, and directly affect the transmission of relevant signals in plants. Crucially, it is now rare to detect these hormones in the metabolites of a single strain (Table 2).

Increasing the activity of key enzymes in plants is an important antioxidant defense mechanism. The key enzymes such as SOD, CAT remove excess reactive oxygen species and protect the plant from salt poisoning [36, 37]. Similarly, a study by Ullah and Bano [38] showed that, salt-tolerant PGPBs isolated from rhizospheric soil of the *Haloxylon salicornicum* also enhanced the growth of salinity stressed maize. These plants exhibited an accumulation of osmolytes (e.g., sugar and proline) and an increase in antioxidant enzyme activity (e.g., SOD, POD, CAT, and ascorbate POD) as compared to uninoculated plants (Fig. 5A, B, C). Sandeep et al. [11] also found that compared with uninoculated seedlings, plants can maintain ion homeostasis, and therefore accumulate less reactive oxygen species and promote plant growth. This is consistent with the results of this study (Fig. 5D, E, F, G).

Abiotic stress usually leads to decreases in leaf water content and affects the content of osmotic regulators [39, 40]. Studies have shown that under abiotic stress, ABA can induce the accumulation of osmoregulators such as proline and soluble sugars [41]. In addition, inoculation with ABA-producing PGPB often decreases the accumulation and concentration of ABA in roots and significantly alters the long distance signaling of shoot-to-root ABA transport in the phloem and the root-to-shoot ABA transport in the xylem [7, 37]; the resulting changes in ABA levels may mitigate the plant's sensitivity to water scarcity. PGPB promote the ability of plants to accumulate compatible solutes to maintain intracellular osmotic balance [10]. Inoculation with the HG-15 strain resulted in an increase in certain osmolytes, such as TSS, total protein content, and a decrease in MDA content. Especially in soil with 0.25% and 0.35% salt concentration, TSS and total protein content of wheat inoculated with HG-15 strain were significantly increased ($p < 0.05$) (Fig. 5B, C). In this study, it was found that *B. subtilis* HG-15 can produce osmotic adjustment factors such as ABA, proline, glutamic acid, and GSH. This improves the water use efficiency of wheat as well as plant salt tolerance [42, 43]. It can be seen from Fig. 8 that Mg, Ca, and K have a positive effect on the accumulation of TSS and total protein in plants, and have a negative correlation with the accumulation of proline, while the result of Na ion was the opposite. The possible reason is that the HG-15 strain inoculation in three wheat strains with different salt concentrations can reduce the stress damage of wheat and affect the proline signal activated under stress, so that it does not change significantly.

The interaction between microorganisms and plant roots is an important factor which promotes nutrient element circulation in soil. Organic matter accumulated by plant photosynthesis is released into the soil through plant roots, providing nutrients and energy for the growth of microorganisms, which decompose insoluble minerals in the soil through their metabolic activities and provide mineral nutrients for the growth of plants [44]. Many microorganisms display strong environmental adaptability, and a large number of functional groups on the surface, such as carboxyl, hydroxyl, amino, amide groups, and other active sites, cause metal ions to be easily adsorbed on the surface of microbial cells [45]. Low molecule organic acids produced by *B. subtilis* can be adsorbed by the soil after complexing with various metal ions such as Cu^{2+} and Na^+ in the soil, thereby reducing the positive charge on soil surface [46, 47]. Beveridge et al. [47] found that cell walls isolated from *Bacillus subtilis* can complex a large number of Mg, Fe, Cu, Na and K. In this study, the HG-15 strain absorbed different amounts of Na, K, and Mg in soils with different salt concentrations (Fig. 2). In the soil with 0.35% salt content, the HG-15 strain complexed more Na (Fig. 2G), reduced the absorption rates of K and Mg ions (Fig. 2H, J), and reduced the relative proportion of Na ions in rhizosphere soil. Under no salt stress, low salt stress, and high salt stress, HG-15 inoculation can reduce soil pH and EC, and increase soil organic matter, total nitrogen, and available nitrogen. The effect of the HG-15 strain on soil available P content was not significant. This may be due not only to the high pH and strong buffer of the soil tested, but also to the limited amount of organic acids produced by the strain. As we all know, phosphorus plays an important role in the improvement of saline-alkali land. Better improvement of saline-alkali soil still requires the addition of nutrients such as external acid phosphate fertilizer.

The main function of K is to enhance the photosynthesis of plants, promote the synthesis of sugar, starch and protein in plants, and improve the disease resistance of crops. Silicate bacteria can release soil potassium, promote the transfer of K from root to leaf, and play an important role in promoting plant nutrient absorption and osmotic pressure regulation [48]. The HG-15 strain has the activity of dissolving potassium, and the enrichment of K in rhizosphere soil is of great help to the growth of plants. Excessive accumulation of Na^+ , which occurs under salt stress, leads to osmotic stress which causes Na^+/K^+ imbalance or even ionic poisoning in plants [49]. As a result, it can decrease the intensity of root injury, cause stomata to close and reduce transpiration tension, and interfere with the ability of the plant body to absorb water, which inhibits its physiological activity and causes a decrease in photosynthetic rate [50]. Eventually, the photosynthetic organs of plants are destroyed and photosynthetic rate is reduced [51, 52]. It is well known that sodium ions can interact with negatively charged plasma membranes, resulting in depolarization of transmembrane potentials and changes in H^+ -ATPase activity, which in turn interfere with the uptake of cations such as Mg^{2+} and affect chlorophyll biosynthesis [53]. The results showed that photosynthesis was stronger in wheat inoculated with HG-15 than in wheat not inoculated with strains (Fig. 4C, E). We speculate that this is due to PGPB changing the ion content in the rhizosphere of plants. The results in Figs. 7 and 8 also confirm that K and Mg in rhizosphere soil have a positive effect on plant photosynthesis. Although HG-15 inoculation did not significantly affect Ca^{2+} in rhizosphere soil (Fig. 2), through correlation analysis (Fig. 8) and RDA analysis (Fig. 7), we can see that Ca^{2+} also has a positive effect on plant growth parameters.

From the results of correlation analysis, available N has the largest positive correlation with biomass accumulation and photosynthesis of plants (Fig. 8). It can be inferred that the available nitrogen content in rhizosphere soil has the greatest influence on plant growth under salt stress. N is the nutrient that most restricts plant productivity. Salt stress can interfere with plant nitrogen nutrition and reduce N content in plant tissues [54]. For example, salt leads to the inhibition of the absorption and assimilation of ammonium and nitrate [55]. Nitrogen-fixing bacteria are an important source of available nitrogen for plants in saline soil. Meanwhile, nitrogen-fixing PGPB can maintain normal cell turgor and metabolism of plants by producing osmotic factors [56].

This study isolated a rhizoplane bacterium *Bacillus subtilis* HG-15 with good growth promoting properties and high salt tolerance (30% NaCl salt concentrations). Through a series of physiological and biochemical tests, scanning electron microscopy (SEM), rep-PCR, and double antibiotic screening techniques, we aimed to analyze the mechanism of growth-promoting and high halotolerant properties of this strain from its genetic characteristics, the change of chemical properties of rhizosphere soil, and the improvement of growth state of wheat. In particular, we detected that the pH of wheat rhizosphere soil inoculated with HG-15 strain was significantly reduced ($P < 0.05$), which is not easy. We speculate that this is related to the large inoculation of the strain and the excellent colonization ability of the strain. Since the strain is isolated from the root surface of the plant, we speculate that in addition to the stable colonizing ability inside the plant and the rhizosphere, it can also produce a large amount of acidic substances. Although these acids did not significantly change the amount of available phosphorus in the soil, they lowered the soil pH (Fig. 2). Different salt-tolerant PGPBs have different PGP characteristics, and different characteristics will cause plants to respond differently [4]. This study confirmed the promotion effect of the HG-15 strain with various PGP characteristics (Table 2) on wheat plant growth under salt stress. Inoculation with this bacterium can regulate the nutrient enrichment and Na^+/K^+ ratio of plant rhizosphere soil, thereby protecting the plant from salt damage. Therefore, our report expands our understanding of the growth-promoting properties of plants in the genus *Bacillus*, especially *Bacillus subtilis*, which is highly salt-tolerant.

Conclusion

The rhizoplane of plants in saline-alkali land may be the reservoir of abundant PGPB, and rhizosporium may have the potential to promote plant growth and colonize the rhizosphere soil and plant interior. *Bacillus subtilis* HG-15 isolated from the rhizoplane of wheat in the Yellow River delta showed prominent salt tolerance and PGP activity in soils under non-salt stress, low salt stress and high salt stress. At the same time, the strain was able to colonize the rhizosphere soil of wheat and the roots, stems and leaves of wheat. The chemical properties of wheat rhizosphere soil were significantly correlated with plant osmotic regulation, oxidation resistance, photosynthesis and biomass accumulation. The effect of PGPB on rhizosphere soil is closely related to plant growth.

Methods

Isolation of bacteria

In May 2016, 12 strains of bacteria were isolated from root surfaces in the wheat rhizoplane in saline-alkali soil of the Yellow River delta in Shandong Province, China (118°49′15″E, 37°24′31″N). The soil parameters were as follows: pH, 8.535; salt content, 0.2143%;

electrical conductivity (EC), 622 $\mu\text{s}/\text{cm}$; organic matter, 25.847 g/kg; total N, 1.67 g/kg; and Olsen-P, 13.42 g/kg. Briefly, to isolate the bacterium, roots (1 g fresh weight) were thoroughly washed, homogenized in 0.5 \times phosphate-buffered saline (PBS; 9 mL), serially diluted to 10^{-7} in sterile nutrient agar (NA) medium with a 4% NaCl concentration, and placed in a 30°C incubator for 48–72 h [57]. The bacterium was subcultured twice. Finally, the isolates were streaked onto NA medium. A glycerol stock solution (30% v/v) of the isolate was prepared and stored at -80°C for later use. Based on colony morphology differences, 12 isolates were identified. Next, we measured the salt tolerance and other PGP activities of these strains to determine the strains with the most potential to promote plant growth in a pot experiment.

Screening for salt tolerance

The isolates were inoculated into Luria-Bertani (LB) medium (1% NaCl) and cultured for 24 h (30°C, 200 rpm/min, 8×10^8 CFU/mL) as seed solutions. The seed solutions were inoculated, in triplicate, into LB medium with different NaCl concentrations (5%, 8%, 10%, 15%, 20%, 25%, 35%) using a 2% inoculum. After 7 d, the absorbances of the cultures were measured with a TU-1810 spectrophotometer (Beijing Puxi General Instruments Co., Ltd., China) at 600 nm. At 30°C, 1 mL of each seed solution was absorbed and diluted to 10^4 [57]. Uninoculated medium was used as a blank control. The isolates were tested for their ability to survive and tolerate salt in water-soluble fertilizer provided by Shandong Agricultural University Fertilizer Co., Ltd. The main components of potassium nitrate-containing humic acid water-soluble fertilizer products were the following: macroelements, ≥ 400 g/L; total N, ≥ 360 g/L; potassium, ≥ 45 g/L; humic acid, ≥ 30 g/L; nitrate N, ≥ 90 g/L; ammonium N, ≥ 90 g/L; and amide N, ≥ 180 g/L. The main components of solid water-soluble fertilizer (sulfuric ammonium yellow silver-humic acid soluble fertilizer) were as follows: $\text{N-P}_2\text{O}_5\text{-K}_2\text{O} = 17\text{-}5\text{-}23$, macroelements, $\geq 45\%$; nitrate N, $\geq 9\%$; and fulvic acid, $\geq 3\%$. The fermentation broth of isolates was inoculated into fertilizer at 5% (v/v) and 5% (w/v) and placed at room temperature for 2 years. Based on salt tolerance of the isolates in LB medium at different NaCl concentrations, we identified HG-15 as the strain with the highest salt tolerance in NA medium. A glycerol stock (15% w/v) of the isolate was prepared and stored at -80°C until further use.

Amplification and sequencing of 16S rRNA genes

To identify the bacterium at the molecular level, its 16S rRNA was amplified by PCR using a standard method [58]. Universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACT-3') were used to amplify the 16S rRNA [59]. Amplified sequences were then gel purified using a TIANquick Midi Purification kit (Tiangen Biotech, China) and sequenced. The obtained 16S rRNA sequence and sequences in NCBI were compared. Next, the pairwise evolutionary distance between the 16S rRNA sequence of the test strain and related bacterial strains was calculated, and a phylogenetic tree was constructed using the neighbor-joining method in MEGA software (version 5.0) [60]. Bootstrapping of 1,000 replicates was used to assess clustering of associated taxa.

Bioassays for the promotion of growth and enhancement of salinity tolerance traits

ACCD assay

The bacterium was first cultured in enriched medium and then transferred to basic medium with ACC as the whole N source. ACCD was determined by quantifying the alpha-ketobutyric acid produced by ACC decomposition. ACCD activity was determined as previously described by Penrose and Glick [61]. The test organism was grown in 15 mL tryptic soy broth to the late logarithmic growth stage and incubated at 30°C for 24 h at 200 rpm. The cells were then harvested via centrifugation, washed with 0.1 M Tris-HCl (pH 7.6), added to 7.5 mL of DF-basic medium containing 3 mM ACC as the sole N source, and cultured at 30°C overnight. ACCD was induced by exposing bacterial cells to an oscillating water bath at a rate of 200 rpm for 24 h at 30°C. Then, the cells were harvested by centrifugation, washed with 0.1 M Tris-HCl (pH 7.6), and resuspended in 600 μL of 0.1 M Tris-HCl (pH 8.5). Next, 30 μL of toluene was added to the cell suspension and shaken for 30 s. Then, 100 μL of toluene cells was used to measure protein and stored at 4°C. The remaining toluene cell suspension was immediately used for the ACCD assay. ACCD activity was determined by measuring the amount of α -ketobutyric acid produced by hydrolytic cleavage of ACC. The amount of α -ketobutyrate (KB) was determined at 540 nm by comparing the absorbance of the test sample with a standard curve of pure KB (Sigma-Aldrich, USA). ACCD activity was expressed as the amount of alpha-KB micron produced per hour in the reaction system.

Nitrogen fixation

To test the ability of the isolate to fix N, a preliminary test of N fixation was conducted by growing the isolate in Ashby's medium devoid of fixed N sources as qualitative evidence of atmospheric N fixation. The ability to fix atmospheric N was evaluated by growth on N-free JNFb medium using a standard protocol [62]. For N-fixing bacteria assays, we measured sugars to determine the N fixation efficacy of

the isolate based on sugar measurement-anthrone photoelectric colorimetry (620 nm) and N measurement-semi-micro photoelectric colorimetry (420 nm). Here, 50 mL of N-free media (1% sugar) was added to a 250 mL flask and sterilized at 121°C for 30 min. After this, two colonies were scraped into individual bottles and shaken at 200 rpm for 3 d at 30°C, and then the bacterial solution was diluted, and the sugar quantity was measured. The sugar standard curve was generated using glucose, whereas the N standard curve was generated using ammonium sulfate. Bacterial N fixation efficiency was considered the mass of N (mg) fixed from the air per 1 g of carbohydrate consumed by the isolate and expressed as mg N/g sugar [63].

Ammonia production

The alkaline solution of mercury iodide and potassium iodide reacts with ammonia to form a reddish-brown colloidal compound. This color is absorbed over a wide wavelength range (410–425 nm). The LB fermentation broth of the isolate was inoculated into 10 mL of peptone solution (peptone, 10 g; NaCl, 10 g; water, 1 L) and then cultured at 37°C for 48 h. Next, 1.0 mL of sodium potassium tartrate solution and 1.5 mL of Nessler reagent were added and mixed. After the mixture was left standing for 10 min, the control was treated with the same amount of sterile LB. Absorbance was measured at 420 nm in a cuvette with a light path of 20 mm, and the amount of ammonia produced was determined according to an ammonium standard curve [64].

Mineral silicate dissolution activity

The strain was cultured in silicate solid medium [sucrose, 10 g; yeast extract, 0.5 g; $(\text{NH}_4)_2\text{SO}_4$, 1 g; Na_2HPO_4 , 2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; CaCO_3 , 1 g; potassium feldspar, 1 g; agar, 15 g; deionized water 1 L]. A clear area was formed around the colony, which indicated that the strain solubilized potassium feldspar [65].

Osmolyte accumulation

Proline in sulfosalicylic acid solution was heated by adding acid ninhydrin, and the solution turned red. After adding toluene, all of the pigments were transferred to toluene, and the depth of the dye indicated the level of proline. This method was modified from one described by Bates et al. [66] to determine proline content. In addition to proline, glutamate acid also plays a role in osmoregulation. It can be extracted with a special reagent, and the results can be determined at 570 nm after adding a chromogenic agent. Reduced glutathione (GSH) is one of the most important anti-oxidation sulfhydryl substances in cells. It plays an important role in anti-oxidation, sulfhydryl protection, and amino acid transport. DTNB reacts with GSH to form a complex with a characteristic absorption peak at 412 nm. Its absorbance is directly proportional to GSH content. GSH concentration can be determined based on the volume of bacterial solution. The content of glutamic acid and GSH was determined using a kit (Solarbio, China).

Phytohormone identification and quantification

IAA reacted with Salkowski's colorifier to change the color of the solution to pink, with a maximum absorption peak at 530 nm. The isolate was inoculated into LB liquid medium for 48 h (30°C, 170 rpm/min). Then, the culture solution was centrifuged at 4°C and $11,292 \times g$ for 5 min, and 0.5 mL of supernatant was taken into a glass tube for the addition of 2.0 mL of Salkowski solution. After 20 min of culturing at room temperature, the absorbance of each tube was measured at 530 nm using a spectrophotometer. The concentration of IAA ($\mu\text{g/mL}$) in the medium was determined by absorbance based on a standard curve [67].

Bacterial cultures (Nfb) in the exponential growth phase were separated into several 20 mL fractions for identification of abscisic acid (ABA), zeatin (ZA), salicylic acid (SA), jasmonic acid (JA), and gibberellins 3 (GA_3). These fractions were centrifuged at $7,200 \times g$ for 20 min at 4°C. The extraction method was as follows: a sample was removed, thawed, shaken, and centrifuged. Next, 1 mL of the supernatant and 0.5 mL of petroleum ether were mixed for triple extraction and decolorization, after which the upper ether phase was discarded, and the lower aqueous phase was adjusted to pH 2–3 using an aqueous 1 mM trichloroacetic acid solution. The triple extraction was performed using an equal volume of ethyl acetate, after which the extract was collected and combined with the ethyl acetate layer, mixed with hydrogen, and diluted with 0.5 mL in the mobile phase. Subsequently, an appropriate amount of solution was removed with a needle filter for testing. The peak area of each standard solution was determined successively according to the chromatographic conditions; the peak area was considered the vertical coordinate and the concentration was considered the horizontal coordinate in calculating the standard curve and correlation coefficient of hormones.

ABA HPLC liquid phase conditions were as follows: mobile phase – mobile phase A was methanol, whereas mobile phase B was 1% aqueous acetic acid in an isocratic elution (50% A + 50% B). ZA HPLC liquid phase conditions were as follows: mobile phase – mobile phase A was methanol, whereas mobile phase B was water in an isocratic elution (30% A + 70% B). SA HPLC liquid phase conditions

were as follows: mobile phase–mobile phase A was methanol, whereas mobile phase B was 1% aqueous acetic acid in an isocratic elution (60% A + 40% B). JA HPLC liquid phase conditions were as follows: mobile phase – mobile phase A was acetonitrile, whereas mobile phase B was 0.1% aqueous phosphate in an isocratic elution (60% A + 40% B). GA₃ HPLC liquid phase conditions were as follows: mobile phase – mobile phase A was methanol, whereas mobile phase B was 1% aqueous acetic acid in an isocratic elution (35% A + 65% B). The computer, detector, and pump were turned on, the column was installed, and the software was opened. Then, in the method group, the injection volume was set to 10 µL with a flow rate of 0.8 mL/min, column temperature of 35°C, sample time of 40 min, and UV wavelength of 254 nm. ABA, ZA, SA, JA, and GA₃ were determined using the corresponding HPLC method at UV wavelengths of 254, 254, 294, 210, and 254 nm.

Screening for other PGP traits

Using yeast dextran as a substrate, the glucanase activity of a bacterial strain can be measured [68]. The amount of reducing sugar in a reaction mixture can be determined using a dinitrosalicylic acid solution [69]. The activity of the iron-producing isolate carrier was determined using chrome azurol S, and growth of the isolate on CAS medium produced an orange halo that was likely due to the activity of an iron carrier [70].

Volatile organic compounds (VOCs) produced by bacteria were determined by GCMS-TQ8050 (X). The fiber extraction head used 65 µm PDMS-DVB fibers, and a Rtx-5MS capillary column (60 m × 0.25 µm ID × 0.25 µm thickness film) with a slope of 5000 was used. The mass spectra of unknown compounds were compared with those in NIST17 and NIST17s (National Institute of Standards and Technology) standard mass spectrometry libraries to determine the structure of the substance corresponding to the peaks.

The pathogenic fungi tested were *Fusarium oxysporum*, *Fusarium pseudograminearum*, *Rhizoctonia solani*, *Fusarium graminearum*, *Botryosphaeria ribis*, and *Botryosphaeria dothidea*. These are all soil-borne pathogenic fungi that cause wheat and other crop diseases [71, 72]. We inoculated pure pathogen cultures, applying each fungus (5 mm disk) to the center of a PDA plate (9 cm in diameter). After culture at 30°C for 24 h, the bacterial isolate was introduced in plates around the fungal cultures, with a distance between the isolate and the center of the dish of 2.5 cm. All plates were cultured in a constant temperature incubator at 30°C for 72 h, and the experiment was repeated three times. The percent growth inhibition was calculated as follows: inhibition (%) = [1 – (fungal growth/control growth)] × 100% [73]. Each experiment was performed in triplicate, and the results were expressed as the mean with standard deviation.

Physiological and biochemical characterization

We used standard protocols for physiological and biochemical tests of our isolate, including Gram stain, starch agar, IMViC (indole, methyl red, Voges-Proskauer, citrate utilization test), and CAT testing [74]. In addition, we used the BIOLOG identification system (BIOLOG Microstation, Biolog Inc, Hayward, CA) for biochemical testing with different carbon sources. The strain was assessed with 71 carbon sources and 23 chemical susceptibility assays according to BIOLOG instructions.

Test of colonization

Colonization of the strain was determined independently using three methods. (1) An HG-15 strain showing rifampicin and spectinomycin resistance was obtained, and the number of colonies in the wheat rhizosphere soil was evaluated on the 7th, 14th, 21st, and 28th day following inoculation. Colony forming units (CFU) per gram of soil were determined using a method previously described by Islam et al. [36]. Each treatment and experiment were replicated three times. (2) The surfaces and cross sections of wheat roots, stems, and leaves were observed via scanning electron microscopy. HG-15 colonization was compared with that on the uninoculated control. (3) According to Singh and Jha [64], rep-PCR gene fingerprints of HG-15 strain and strains recovered from wheat rhizosphere soil, roots, stems, and leaves indicates colonization. We used a rep-PCR reaction mixture containing 25 µL 10 × Taq PCR master mix, 1 µL template DNA, 2 µL of BOX-AIR primer (CTACGGCAAGGCGACGCTGACG) (Sangon, China), and 22 µL of ddH₂O. PCR amplification was performed under the following conditions: initial denaturation at 95°C for 7 min; 35 cycles of 94°C for 1 min, 53°C for 1 min, and 65°C for 8 min; and a final extension at 65°C for 16 min. (4) The surface of wheat was sonicated, treated with 75% alcohol for 2 min and 2% sodium hypochlorite for 10 min for disinfection, and finally washed five times with sterile water. Treated roots, stems, and leaves were cut separately into small pieces of approximately 0.5 cm, and tissue sections were brought into contact with PDA medium containing rifampicin and spectinomycin. The water in the final wash step was cultured to test the thoroughness of sterilization. The culture was carried out at 30°C for 3–6 d; the noninoculated wheats were used as the control. In this manner the HG-15 colonization of wheat roots, stems, and leaves was detected.

Pot experiments

The soil used for potted plants was obtained at 0–20 cm of depth in wheat fields in the Yellow River delta (118°41'07"E, 37°17'17"N) (Dongying City, Shandong, China) in October 2018. The soil was brought to the greenhouse and passed through a 0.5 cm sieve. The number of bacteria cultured from the sample was 1.36×10^4 CFU·g⁻¹ of dry weight soil. The upper and lower inner diameters of the flowerpot were 16.5 cm and 12 cm, respectively. The soil in each pot weighed 2.2 kg. The soil was also analyzed for its nutrient content. Various soil parameters were as follows: pH, 8.329; salt content, 0.1492%; EC, 456 $\mu\text{S}/\text{cm}$; organic matter, 23.51 g/kg; total N, 1.072 g/kg; Olsen-P, 0.0104 g/kg; K⁺, 0.6782 g/kg; Na⁺, 1.0162 g/kg; Ca²⁺, 0.23863 g/kg and Mg²⁺, 0.50805 g/kg.

The HG-15 strain was inoculated in LB liquid medium at 200 rpm and 30°C for 12 h (logarithmic growth period). The bacterial suspension was then centrifuged at $1,073 \times g$ for 10 min to harvest cells and resuspended immediately in sterile water three times. The HG-15 suspension (1×10^8 CFU/mL) was adjusted to its final concentration with sterile water. The experimental wheat variety used was Jimai 21 (The crop research institute of Shandong Academy of Agricultural Sciences considered (865168 / nongda 84-1109) F1 as the mother parent and ji 84-5418 as the father for sexual hybridization and systematic breeding). Wheat seed surfaces were sterilized with 70% ethanol (v/v) for 2 min and washed with disinfectant water three times. Seeds were then placed in 1% (w/v) NaClO solution for 3 min and rinsed with sterile water three times to remove residual sodium hypochlorite [64]. A total of 72 pots were planted with 10 wheat seedlings in each pot. After 7 d, the above ground height of the wheat was approximately 5 cm; wheat with greater or less height was removed. In the treatment group, 20 mL of cell suspension was applied; in the control group, 20 mL of water was used instead. Plants were irrigated with NaCl solution for salt stress (0.15% 456 $\mu\text{S}/\text{cm}$, 0.25% 722 $\mu\text{S}/\text{cm}$, 0.35% 972 $\mu\text{S}/\text{cm}$). Inoculated and uninoculated bacteria were introduced into 12 pots for each concentration. The pots were placed in a completely random design, with each treatment repeated three times. The position of the pot was changed randomly during the trial to eliminate environmental errors. Greenhouse conditions were 20–28°C, 45-50% humidity, and natural lighting. The planting cycle was 28 d. The plants were watered thoroughly at sowing, and then watered once a week with an equal amount (300 mL) of water in each pot. The plants were not watered at 22–28 d. No nutrient solution or other fertilizer was added during the growth cycle. The combined weight of root and shoot was taken as the fresh weight of each plant. The dry weight of the plant was measured after placing a plant in the oven at 70°C for 2 d. For accuracy and precision, each sample was tested in triplicate. The growth of plants was measured in terms of root length, plant height, fresh weight, and dry weight.

Effect of HG-15 on soil chemistry properties under NaCl stress conditions

Soil pH and EC values were analyzed with 1:2.5 and 1:5 soil-water ratios using digital pH (FE20) and EC (FE930) meters (Mettler Toledo, Switzerland), respectively. Organic carbon content was determined using 1 N potassium dichromate for titration and 0.5 N ferrous ammonium sulfate for back titration [75]. Olsen P was extracted with 0.5 M NaHCO₃ and determined according to the procedure described by Olsen et al. [76]. Total N was measured using the Bremner [77] method.

Bulk soil was shaken off by uprooting the wheat. Soil still adhering to the root surface (concentrated within 2 mm of the root surface) was considered rhizosphere soil. We used a sterilized brush to collect the soil in a sterile bag on an ultra-clean worktable. For ion analysis, 0.2 g of wheat rhizosphere soil was treated with 1 mL deionized water and 5 mL concentrated sulfuric acid overnight, and then the cooked liquid was fixed to 50 mL. Measurements were carried out on 1 mL of the solution, which was extracted and diluted 10 times. Na⁺, K⁺, Ca²⁺ and Mg²⁺ content was measured via inductively coupled plasma optical emission spectroscopy (ICP, Thermo Scientific™ iCAP™ 7000 Plus, USA) [64].

Effects of HG-15 on plant growth under NaCl stress conditions

Photosynthetic characteristics and soluble sugar and proline content

Fresh leaves were treated overnight with absolute ethanol, and then chlorophyll (Chl) and carotenoids were extracted and measured spectrophotometrically using the method described by Arnon [78]. Leaf soluble sugar was extracted from boiling water and quantified via the method by Thomas [79]. Proline content was determined according to the method described by Bates [80], wherein valine was extracted with 3% sulfosalicylic acid and filtered. Next, an aliquot of the filtrate was supplemented with 1 mL ninhydrin and glacial acetic acid reagent. The mixture was then boiled for 1 h, placed on ice to stop the reaction, and the absorbance of the sample was measured at 520 nm using a UV spectrophotometer.

Net photosynthetic rate (A), stomatal conductance (g_{sw}), intercellular CO₂ concentration (C_i), and transpiration rate (E) of fully expanded leaves were analyzed using a LI-6800XT (Li-Cor, USA) portable photosynthetic apparatus after 28 d of treatment. The measurement time was 9:00–11:00, and the photon flux was set to 1200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Five leaves were examined for each treatment. Following an

assessment of photosynthetic characteristics, chlorophyll fluorescence parameters were determined using an IMAGING-PAM (Walz, Effeltrich, Germany) fluorometer, and PSII primary light energy conversion efficiency was defined as F_v/F_m . Before measurement, the leaves were dark-adapted for 20 min, and three leaves were selected for each treatment. All operations were carried under background light intensity conditions less than $1 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The intensity of saturated pulsed light of the instrument was $2400 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and that of the measured light was less than $0.5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ [82].

Biochemical analysis for osmolytes in plants after NaCl and bacterial inoculation

Approximately 0.2 g of fresh leaves was placed in a precooled mortar. First, 1 mL of 50 mmol/L buffer (containing 2% polyvinylpyrrolidone, pH 7.8, 4°C) was added, and the leaves were ground into a homogenate in an ice bath. Then, we washed the mortar with 0.5 mL of the above buffer solution to reach a final volume of 1.5 mL. After centrifugation at $12,000 \times g$ and 4°C for 20 min, the supernatant was considered the crude enzyme solution.

In the presence of H_2O_2 , POD can oxidize guaiacol to form a tan substance, which can be measured by a spectrophotometer. We removed 200 mL of PBS (0.2 mol/L, pH 6.0), and 0.076 mL guaiacol (2-methoxyphenol) was added to the liquid, heated, and stirred to allow it to dissolve. After cooling, 0.112 mL 30% H_2O_2 was added, and the mixture was used as the reaction solution. Three milliliters of reaction solution were added to 10 μL crude enzyme solution, and 10 μL PBS was added to 3 mL reaction solution in the control group. POD activity was represented by $\Delta\text{OD}_{470}/(\text{mg}\cdot\text{min})$.

Using SOD in the presence of oxidized substances, riboflavin can be reduced by light. The reduced riboflavin is easily reoxidized under aerobic conditions to produce O_2 , which can reduce nitro blue tetrazolium to a blue methyl trace, which has a maximum absorption at 560 nm. SOD can remove O_2 and inhibit the formation of methyl hydrazone. The enzyme activity can be calculated by photoreduction. We prepared a phosphoric acid buffer (0.05 mol/L, pH 7.8), with 3.1 mL used in the treatment group and 3.2 mL in the blank group. Then, we added 1 mg/mL EDTA- Na_2 (0.2 mL), 20 mg/mL L-methionine (0.2 mL), 0.1 mg/mL riboflavin solution (0.2 mL), and 1 mg/mL nitrogen blue tetrazole solution (0.2 mL) to each group. In the treatment group, 0.1 mL crude enzyme solution was added, whereas, in the blank group, no enzyme solution was added. The total volume of the reaction was 4 mL. The first group was treated with light for 30 min (4000 Lx), and the reaction was terminated with darkness. The second group was treated with darkness for 30 min. The OD_{560} was measured in the second group. The activity of SOD was based on 50% inhibition of photoreduction of nitroblue tetrazolium as an enzyme activity unit (U), and the activity of SOD was expressed as $\text{U}\cdot\text{mg}^{-1}$ [82].

CAT activity was determined based on ultraviolet absorption. H_2O_2 has strong absorption at 240 nm wavelength, CAT can decompose hydrogen peroxide, and the absorbance (A_{240}) of reaction solution decreases with reaction time. The activity of CAT can be measured according to the rate of change in absorbance. We obtained 200 mL of PBS (0.15 mol/L, pH 7.0), added 0.3092 mL 30% H_2O_2 , and shook the solution well. We then obtained 3 mL of reaction solution and added 50 μL of crude enzyme solution, (50 μL of PBS was added to the control group with 3 mL of reaction solution), with the absorbance at 240 nm read once every 1 min for 2 min in total. An extinction coefficient of $39.4 \text{ mM}^{-1}\cdot\text{cm}^{-1}$ for H_2O_2 at 240 nm was used to calculate activity. The enzyme content of A_{240} decreased by 0.1 in 1 min was regarded as the enzyme activity unit ($\text{U}\cdot\text{mg}^{-1}$) [82].

Malondialdehyde (MDA) is one of the most important products of membrane lipid peroxidation. Under high temperature, MDA can react with thiobarbituric acid to form trimethyl Sichuan (3,5,5-trimethyloxazole 2,4-dione), which has an absorption peak at 532 nm and a smaller light absorption at 600 nm. According to the extinction value at 532 nm, the content of MDA in a solution can be calculated. We obtained 0.1 g of plant sample, added 3 mL of 10% TCA for grinding, and centrifuged the sample at $3000 \times g$ for 10 min. We then then obtained 2 mL of supernatant, added 2 mL of 0.65% TBA solution, mixed well, boiled it in water for 15 min and cooled it quickly, measured the supernatant at OD_{440} , OD_{532} , and OD_{600} , and reported the result as $\mu\text{mol}\cdot\text{g}^{-1}$ Pro [83].

Statistical analysis

Data analysis was performed using IBM SPSS 19.0. The Q-Q plot method was used to show that soil and plant parameters were normally distributed. For the same salt stress test with different treatments, t-testing ($p < 0.05$) was used. One-way ANOVA and Dunnett test were used to analyze the data under different salt stress conditions. A redundancy analysis (RDA) of soil and plant parameters was performed using Canoco 4.5.1 (Microcomputer Power, Ithaca, USA) software, and factors with significant explanatory functions were tested with conditional term effect analysis. Pearson test (two-tailed) was used to analyze the correlation between soil and plant indexes, and the results were made into a heat map using Origin 9.0 software.

Abbreviations

N: Nitrogen; AN: Available N; OM: Organic matter; TN: Total N; OP: Olsen-P; EC: Electrical conductivity; PRO: Proline; TSS: Total soluble sugar; TP: Total protein; MDA: Malondialdehyde; POD: Peroxidase; SOD: Superoxide dismutase; CAT: Catalase; Chl a: Chlorophyll a; Chl b: Chlorophyll b; *A*: Net photosynthetic rate; *G_{SW}*: Stomatal conductance; *E*: Transpiration rate; *C_i*: Intercellular CO₂ concentration; *F_v/F_m*: PSII primary conversion efficiency; DW: Total dry weight of root and shoot; FW, Total fresh weight of root and shoot; Height: Plant height; RL: Root length; PGPB: Plant growth-promoting bacteria; *B. subtilis*: *Bacillus subtilis*; PGP: Plant growth-promoting; IAA: Indole-3-acetic acid; ACCD: 1-aminocyclopropane-1-carboxylic acid deaminase; EPS: Extracellular polysaccharides; VOCs: Various volatile organic compounds; IST: Induced systemic tolerance; HPLC: High Performance liquid chromatography; NIST: National institute of Standards and Technology; ABA: Abscisic acid; ZA: Zeatin; SA: Salicylic acid; JA: Jasmonic acid; GA3: Gibberellins 3. PBS: Phosphate-buffered saline.

Declarations

Conflict of interest statement

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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Not applicable.

Author contributions

CJ and HT conceived and designed the experiment; CJ, HT, XW, LH, CW and YZ performed the experiment; CJ, HT, RX, XS, and YL analyzed the data; CJ wrote the paper. XLL guided the research work and revised the manuscript. CJ and HT contributed equally to this work. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed, during current study are available and can be obtained from the corresponding author and first author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no competing interests.

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Tables

Table 1 | Biochemical and physiological characteristics of the HG-15

Characteristic(s)	Activity	Carbohydrate	Utilization	Carbohydrate	Utilization	Chemical Sensitivity Assays	Reaction
Grams stain	∅	α-Cyclodextrin	∅	Glycerol	∅	Troleandomycin	∅
Glucose oxidative fermentation	Fermentation type	Dextrin	∅	γ-Aminobutyric Acid	∅	Rifamycin SV	∅
Aerobism test	Facultative anaerobic	Glycogen	∅	L-Serine	∅	Minocycline	∅
Catalase test	∅	D-Mannitol	∅	D-Serine	∅	Lincomycin	∅
Nitrate reduction	∅	D-Melibiose	∅	L-Phenylalanine	∅	Guanidine HCl	∅
Starch hydrolysis	∅	D-Psicose	∅	L-Ornithine	∅	Vancomycin	∅
V-P test	∅	Acetic Acid	∅	Hydroxy-L-Proline	∅	Tetrazolium Violet	∅
Salt tolerance 5% NaCl	∅	Formic Acid	∅	Bromosuccinic Acid	∅	Tetrazolium Blue	∅
Salt tolerance 10% NaCl	∅	Succinamic Acid	∅	Citric Acid	∅	Nalidixic acid	∅
Citrate assimilation test	∅	L-Leucine	∅	L-Histidine	∅	Sodium Bromate	∅
Methyl red test	∅	L-Fucose	∅	Uridine	∅	Potassium Tellurite	∅
Phenylalanine amino acid deaminase	∅	α-D-Lactose	∅	L-Threonine	∅	1% Sodium Lactate	∅

+ positive, – negative

Table 2 | Plant growth-promoting effects of *Bacillus subtilis* HG-15 strain on wheat traits

Plant growth promoting traits	Activity
Salt tolerance	30%
Liquid water soluble fertilizer (2 years)	82.63%
Solid water soluble fertilizer (2 years)	85.37%
ACCD production	14.816 ± 0.965 µmol/(mg·h)
1AA production	154.53 ± 4.17 µg/mL
ABA production	0.609 ± 0.026 µg/mL
GA ₃ production	0.103 ± 0.005 µg/mL
ZA production	0.638 ± 0.014 µg/mL
JA production	0.430 ± 0.016 µg/mL
SA production	3.865 ± 0.098 ng/mL
Proline production	19.862 ± 0.4748 µg/mL
Glutamic acid production	33.488 ± 1.066 mg/mL
GSH production	0.187 ± 0.006 µmol/mL
Siderophore production	+
Ammonia production	13.577 ± 0.530 µg/mL
Nitrogen fixation	24.304 ± 0.7536 mg N/g glucose
Potassium solubilizing	+
Xylanase	76.813 ± 2.097 u/mL
VOCs	2,3-Butanediol, [R-(R*,R*)]-/ 2,3-Butanediol / 2-Heptanone/ 2-Nonanone / 2-Nonanol / 2-Dodecanone / Pentadecane/ Heptadecane

Note: Values are mean values ± standard deviations. Uninoculated bacteria were used as negative control. + positive, – negative

Table 3 | Population density of rhizosphere colonization of wheat by HG-15 strain in soil with different salt concentrations (10⁶ CFU·g⁻¹ dry weight of soil)

Salinity conditions	Days after inoculation (d)			
	7	14	21	28
0.15%	31.40 ± 2.99a	25.36 ± 2.39b	4.20 ± 1.57c	2.38 ± 0.72c
0.25%	9.50 ± 1.61a	6.37 ± 1.60b	0.76 ± 0.15c	0.57 ± 0.05c
0.35%	4.41 ± 1.65a	1.65 ± 0.72b	0.22 ± 0.08b	0.15 ± 0.02b

0.15%, 0.25% and 0.35% respectively represent the soil with different salt concentrations used in the pot experiment. Values are expressed as mean ± standard deviation. Statistical analysis was accomplished with a one-way ANOVA followed by the Duncan test. Means sharing a common letter within the same row are not significantly different at $p < 0.05$.

Table 4 | The main and interaction effects of both salinity and bacterium on measured parameters.

	pH		EC		Organic matter		Olsen-P		Total N		Available N		Na		K		Ca		Mg	
	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
HG-15	68.535	0.000**	145.997	0.000**	908.604	0.000**	6.017	0.030*	167.186	0.000**	48.793	0.000**	33.947	0.000**	17.668	0.001**	7.003	0.021*	47.294	0.000**
Salt	15.305	0.000**	6316.708	0.000**	308.999	0.000**	3.817	0.052	162.139	0.000**	82.217	0.000**	266.133	0.000**	21.402	0.000**	11.735	0.001**	25.205	0.000**
Salt	4.078	0.045*	24.481	0.000**	75.668	0.000**	0.517	0.609	12.810	0.001**	2.913	0.093	2.537	0.120	1.415	0.281	0.168	0.847	1.018	0.391
x																				
HG-15																				
	dry weight		fresh weight		plant height		root length		A		gsw		E		Ci		Chl a		Chl b	
	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
HG-15	213.390	0.000**	65.135	0.000**	213.390	0.000**	65.135	0.000**	11.917	0.005**	29.032	0.000**	19.478	0.001**	2.268	0.158	25.887	0.000**	12.366	0.004**
Salt	155.056	0.000**	72.974	0.000**	155.056	0.000**	72.974	0.000**	33.375	0.000**	66.938	0.000**	96.001	0.000**	10.053	0.003**	36.902	0.000**	33.775	0.000**
Salt	14.222	0.001**	4.887	0.028*	14.222	0.001**	4.887	0.028*	0.835	0.458	5.049	0.026*	1.663	0.230	0.901	0.432	5.425	0.021*	0.491	0.624
x																				
HG-15																				
	Fv/Fm		Proline		TSS		Total protein		MDA		POD		SOD		CAT					
	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P				
HG-15	2.354	0.151	273.491	0.000**	198.372	0.000**	171.471	0.000**	327.578	0.000**	7.205	0.020*	0.829	0.380	10.479	0.007**				
Salt	23.968	0.000**	449.485	0.000**	117.815	0.000**	112.776	0.000**	140.613	0.000**	91.949	0.000**	21.897	0.000**	338.937	0.000**				
Salt	0.622	0.553	104.857	0.000**	7.318	0.008**	4.670	0.032*	2.468	0.127	0.934	0.420	0.481	0.630	0.771	0.484				
x																				
HG-15																				

The interaction effect sizes of bacteria and different soils on rhizosphere soil parameters and plant parameters based on two-way ANOVA with 95% confidence intervals. Data in the table are p values. * means $p < 0.05$, ** means $p < 0.01$. dry weight, total dry weight of root and shoot; fresh weight, total fresh weight of root and shoot; A, net photosynthetic rate; gsw, stomatal conductance; E, transpiration rate; Ci, intercellular carbon dioxide concentration; Chl a, Chlorophyll a; Chl b, Chlorophyll b; TSS, soluble sugar; MDA, malondialdehyde; POD, peroxidase; SOD, superoxide dismutase; CAT, catalase. Bold indicates that there is a significant interaction between bacteria and soil

Supplemental Legends

Figure S1 | BOX-PCR profile of bacterium colonized in wheat plants and confirmation of its identity using pure culture. Lane M: DNA marker, Lane RS: DNA of *B. subtilis* isolated from rhizosphere soil of inoculated plants, Lane R: DNA of *B. subtilis* isolated from roots of inoculated plants, Lane S: DNA of *B. subtilis* isolated from rhizosphere stems of inoculated plants, Lane L: DNA of *B. subtilis* isolated from leaves of inoculated plants, Lane HG-15: control DNA.

Figure S2 | In vitro antagonistic activity of *B. subtilis* HG-15 strain against selected pathogen in dual culture assays on PDA medium, 72h after incubation. (A) *Fusarium oxysporum*, (B) *Fusarium pseudograminearum*, (C) *Rhizoctonia solani*, (D) *Fusarium graminearum*, (E) *Botryosphaeria ribis*.

Figures

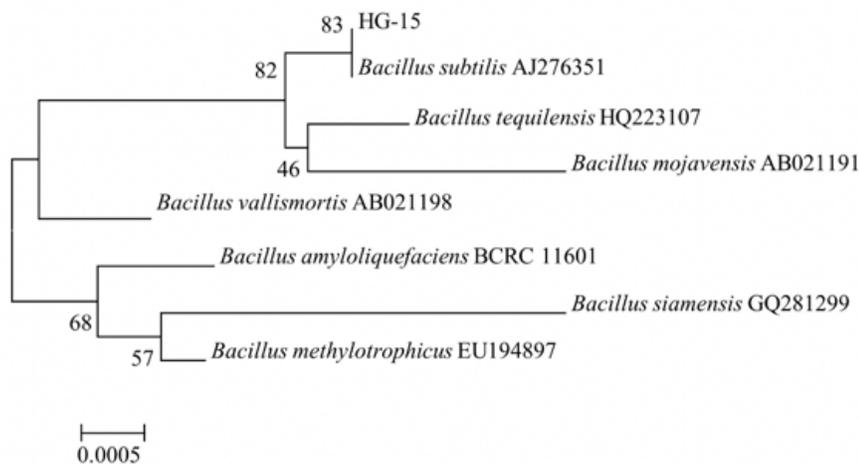


Figure 1

Phylogenetic tree showing relationships among bacteria associated with *B. subtilis* HG-15. The 16S rRNA gene sequence (1452 bp) of closely related strains was obtained from the NCBI GenBank database. The tree was generated at a bootstrap value ($n = 1000$) using the Neighbor joining from Mega 5.0 software.

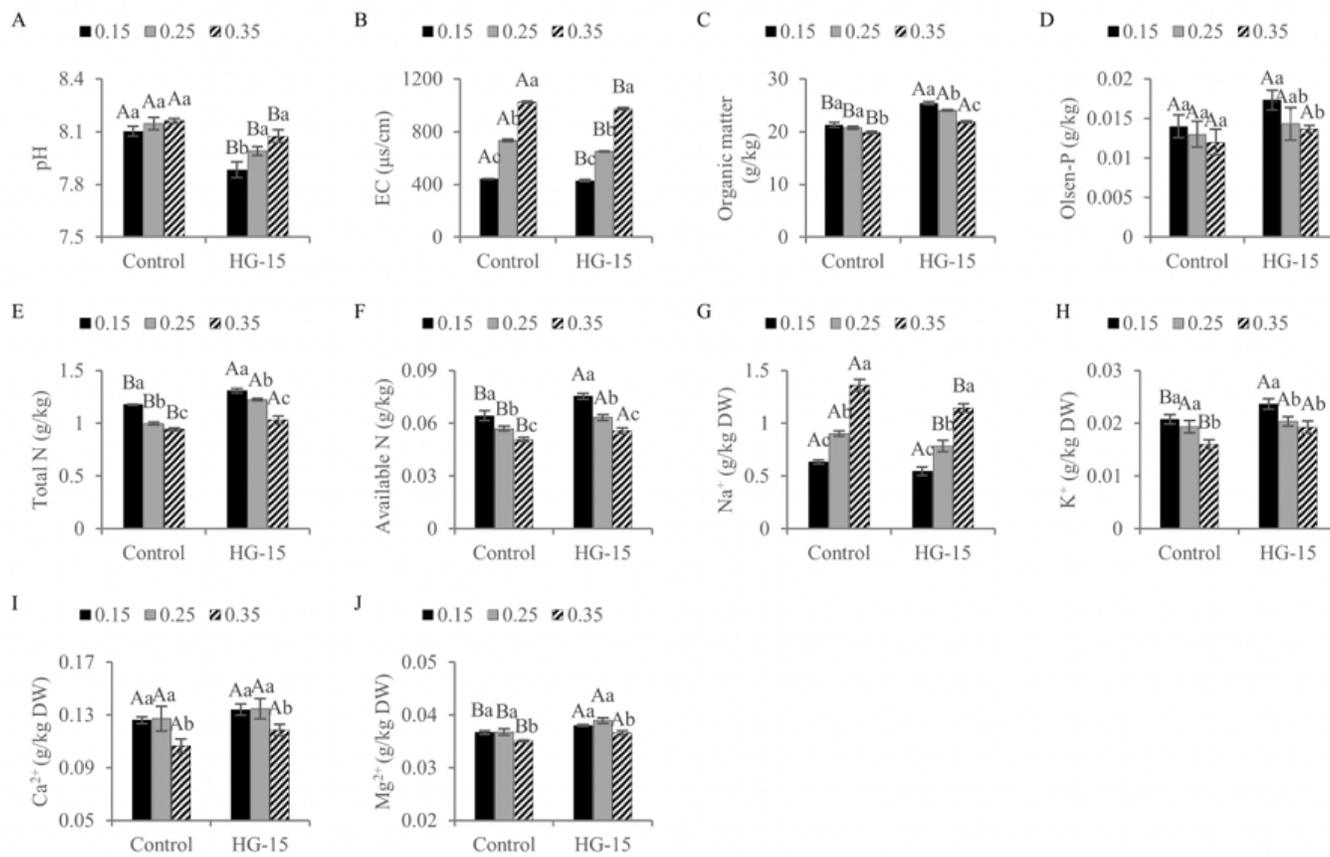


Figure 2

Effects of *B. subtilis* HG-15 inoculation on physical and chemical properties of soil under different salinity conditions (0.15%, 0.25%, 0.35% NaCl). (A) pH, (B) EC, (C) Organic matter, (D) Olsen-P, (E) Total N, (F) Available N, (G) Na⁺, (H) K⁺, (I) Ca²⁺, (J) Mg²⁺. Data shown are the means ± standard deviations (n = 5). Capital letters indicate the difference between CK and HG-15 inoculation under the same salt concentration stress (Student's t-test, p < 0.05), whereas lowercase letters indicate differences in three different salt concentration stresses in the control (or HG-15) group (one-way ANOVA, p < 0.05), respectively.

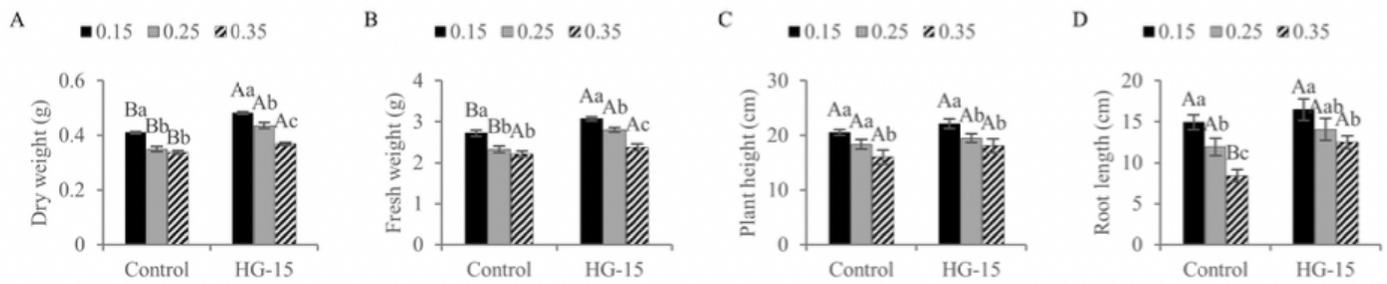


Figure 3

Effect of *B. subtilis* HG-15 inoculation on plant biomass under different salinity conditions (0.15%, 0.25%, 0.35% NaCl). (A) Dry weight, (B) Fresh weight, (C) Plant height, (D) Root length. Data are means ± standard deviations (n = 5). Capital letters indicate the difference between CK and HG-15 inoculation under the same salt concentration stress (Student's t-test, p < 0.05), whereas lowercase letters indicate differences in three different salt concentration stresses in the control (or HG-15) group (one-way ANOVA, p < 0.05), respectively.

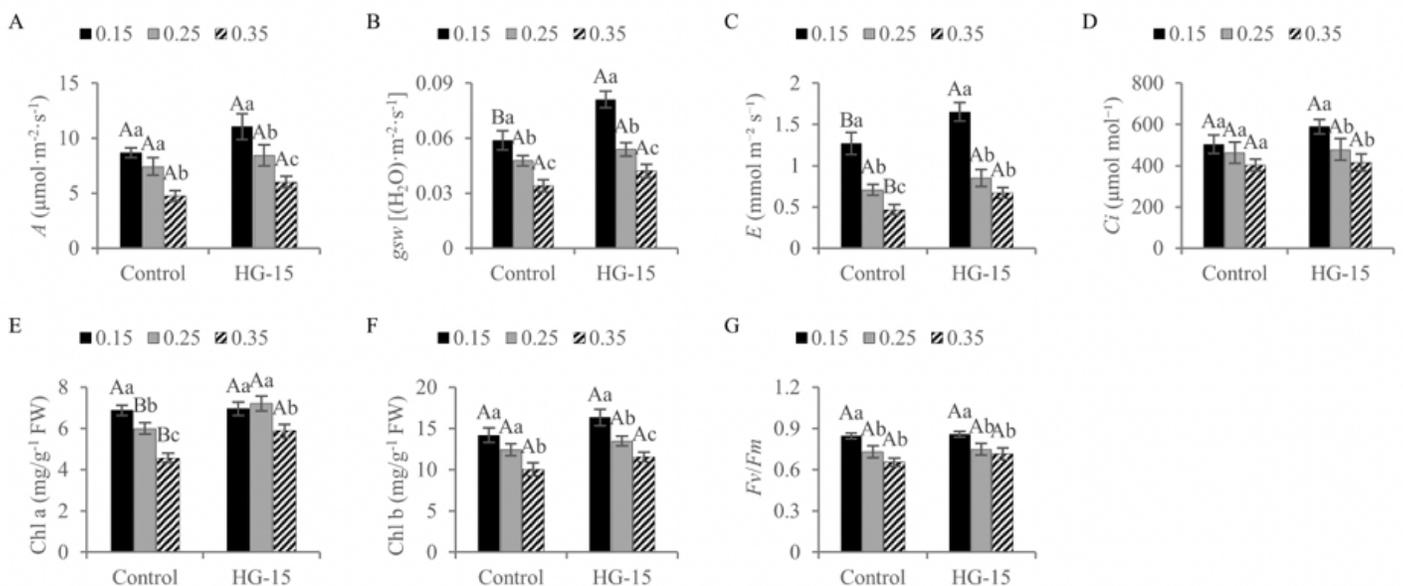


Figure 4

Effects of *B. subtilis* HG-15 inoculation on chlorophyll content and photosynthesis of plants under different salinity conditions (0.15%, 0.25%, 0.35% NaCl). (A) Chlorophyll A, (B) chlorophyll B, (C) net photosynthetic rate (A), (D) stomatal conductance (g_{sw}), (E) transpiration rate (E), (F) intercellular CO₂ concentration (C_i), (G) F_v/F_m. Data are the means ± standard deviations (n = 5). Capital letters indicate the difference between CK and HG-15 inoculation under the same salt concentration stress (Student's t-test, p < 0.05), whereas lowercase letters indicate differences in three different salt concentration stresses in the control (or HG-15) group (one-way ANOVA, p < 0.05), respectively.

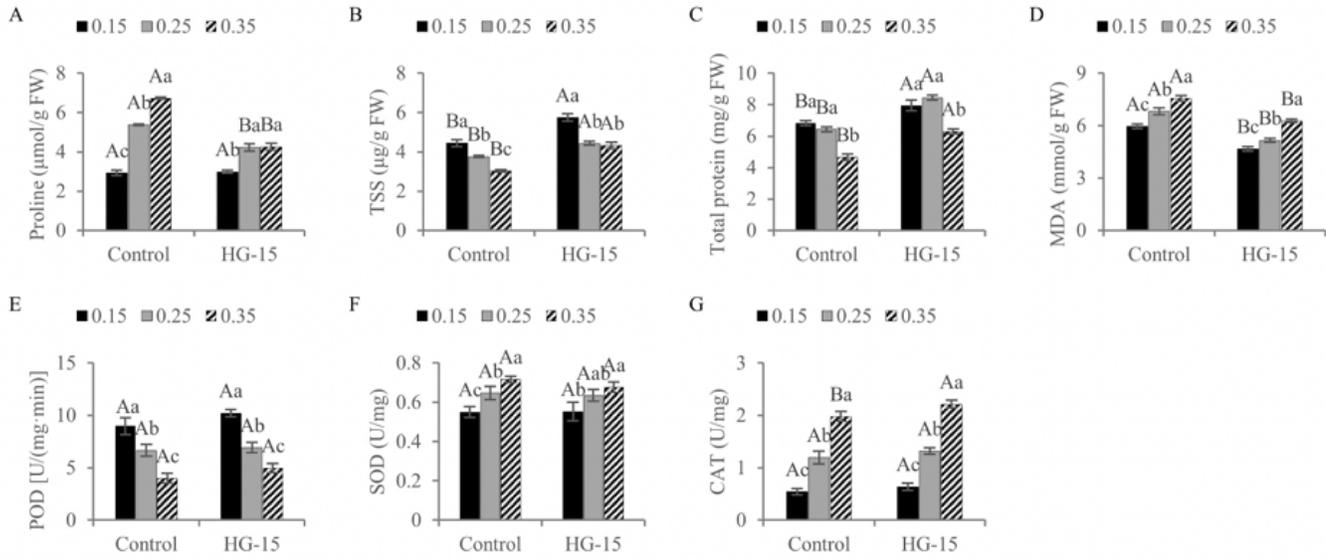


Figure 5

Effect of *B. subtilis* HG-15 inoculation on (A) Proline, (B) TSS, (C) Total protein (D) MDA, (E) POD, (F) SOD, (G) CAT under different NaCl salinity conditions. Data are means ± standard deviations (n = 5). Capital letters indicate the difference between CK and HG-15 inoculation under the same salt concentration stress (Student's t-test, p < 0.05), whereas lowercase letters indicate differences in three different salt concentration stresses in the control (or HG-15) group (one-way ANOVA, p < 0.05), respectively.

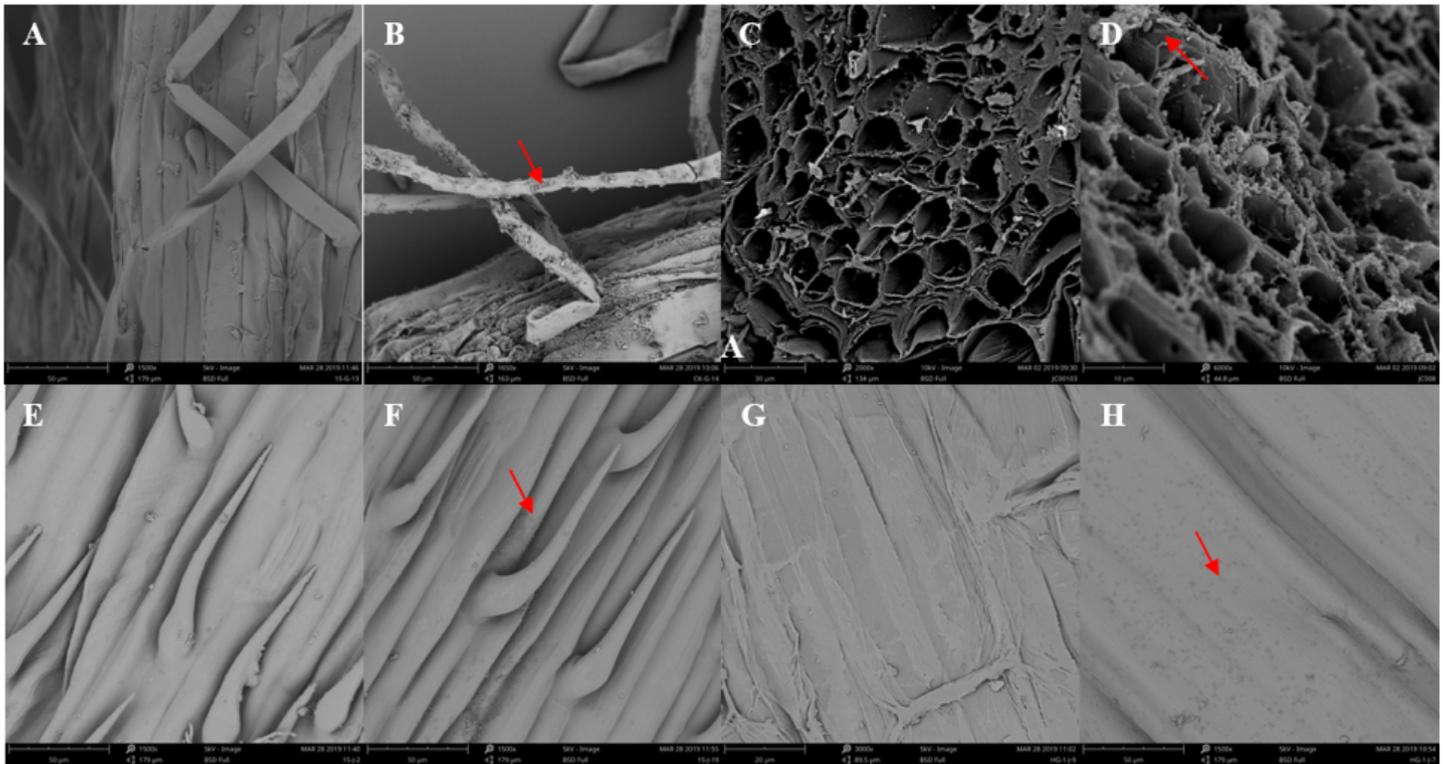


Figure 6

Scanning electron micrographs of control and +HG-15 in 7 d old wheat seedlings. (A-B) Root surface, (C-D) root, (E-F) leaf surface, (G-H) stem surface. Control (uninoculated) is shown on the left and the inoculated HG-15 treatment (+HG-15) is shown on the right. Note the micrographs show large number of bacterial colonies present in the column.

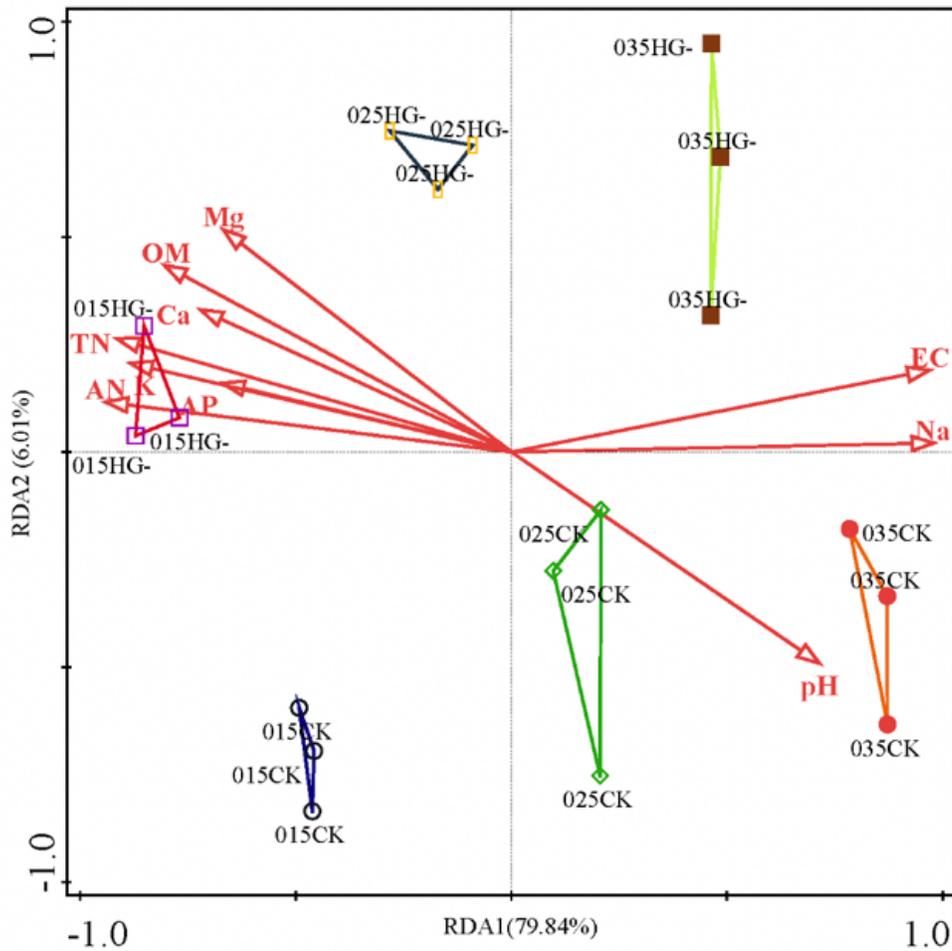


Figure 7

RDA analysis of different soil parameters and plant parameters. 0.15CK: plant parameters in 0.15% salt concentration; 0.25CK: plant parameters in 0.25% salt concentration; 0.35CK: plant parameters in 0.35% salt concentration; 0.15HG-: plant parameters after inoculation of HG-15 strain at a salt concentration of 0.15%; 0.25HG-: plant parameters after inoculation of HG-15 strain at a salt concentration of 0.25%; 0.35HG-: plant parameters after inoculation of HG-15 strain at a salt concentration of 0.35%; Mg: Mg²⁺ concentration; OM: organic matter; Ca: Ca²⁺ concentration; TN: total N; AN: available N; AP: Olsen-P; EC: electrical conductivity; Na: Na⁺; pH: soil pH.

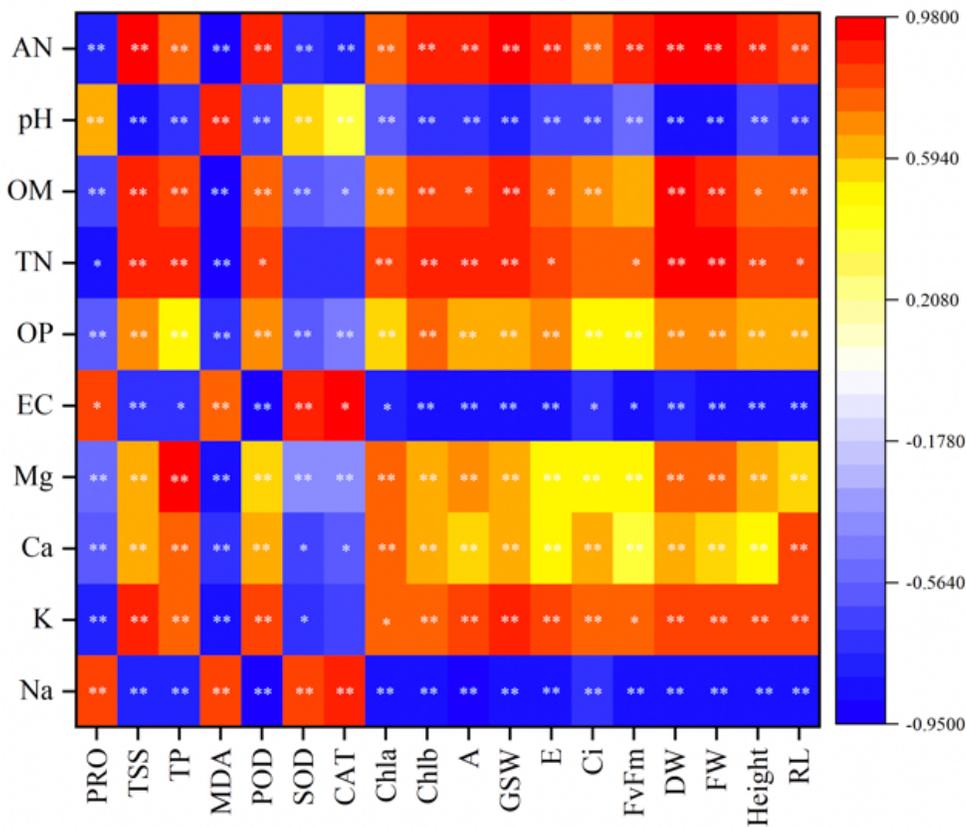


Figure 8

Correlation analysis of soil parameters and plant factors under the effect of HG-15 strain. AN: Available N; pH: soil pH; OM: Organic matter; TN: Total N; OP: Olsen-P; EC: Electrical conductivity; Mg: Mg²⁺ concentration; Ca: Ca²⁺ concentration; K: K⁺ concentration; Na: Na⁺; PRO: Proline; TSS: Total soluble sugar; TP: total protein; MDA: malondialdehyde; POD: peroxidase; SOD: superoxide dismutase; CAT: catalase; Chl a: chlorophyll a; Chl b: chlorophyll b; A: net photosynthetic rate; GSW: stomatal conductance; E: transpiration rate; Ci: intercellular CO₂ concentration; Fv/Fm: PSII primary conversion efficiency; DW: total dry weight of root and shoot; FW, total fresh weight of root and shoot; Height: plant height; RL: root length. Blue indicates negative correlation between parameters and red indicates positive correlation between parameters. *, p < 0.05; **, p < 0.01.

Supplementary Files

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