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The biocontrol strain *Bacillus mojavensis* KRS009 confers resistance to cotton Verticillium wilt and improves tolerance to salt stress

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Abstract

Biological control has gained increasing attention as a strategy to address biotic and abiotic stresses in crops. In this study, we identified the strain KRS009 as *Bacillus mojavensis* through morphological identification and multilocus sequence analysis. KRS009 exhibited broad-spectrum antifungal activity against various phytopathogenic fungi by secreting soluble and volatile compounds. Additionally, the physio-biochemical traits of strain KRS009 were characterized, including its growth-promoting capabilities and active enzymes. Notably, KRS009 demonstrated the capacity for biofilm formation and exhibited tolerance to saline-alkali conditions. The biological security evaluation confirmed the safety of KRS009 for both humans and plants. Furthermore, strain KRS009 was found to trigger plant immunity by inducing systemic resistance through salicylic acid- and jasmonic acid-dependent signaling pathways. Greenhouse experiments conducted on cotton plants proved that the treatment with strain KRS009 effectively protected cotton against Verticillium wilt caused by *Verticillium dahliae* and promoted the growth of cotton under salt stress. These findings highlight the potential of *B. mojavensis* KRS009 as a promising biocontrol and biofertilizer agent for promoting plant growth, combating fungal diseases and mitigating salt stress in plants.

Fuhua Zhao and Dan Wang contributed equally to this paper.

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KEYWORDS
Bacillus mojavensis KRS009, biocontrol, plant immunity, salt stress, verticillium wilt

1 | INTRODUCTION

Cotton (*Gossypium*) is a vital renewable resource, renowned as the primary fiber crop, as well as its environmental and social benefits.[1] However, cotton plants face various biological and abiotic stresses that significantly impact crop yield. Cotton Verticillium wilt caused by *Verticillium dahliae* is one of the major threats to cotton production worldwide. As a soilborne vascular disease, cotton Verticillium wilt is widely distributed and highly destructive, causing substantial economic losses for the cotton industry. Abiotic stresses, including high temperatures, drought, and salinity, impose significant restrictions on cotton growth and productivity, especially salt stress.[2,3] There were over 953 million hectares of saline-alkali land in China, of which Xinjiang accounted for 22.1% of this area, totaling 2.2×10^7 ha.[4,5] Xinjiang is one of the provinces with the largest and most concentrated saline-alkalidistribution area and the most serious saline-alkali threat in northwest China, and there are substantial challenges in ameliorating these conditions.[6] The widespread distribution of saline-alkali land in major cotton-producing regions affects both the quality and yield, leading to substantial economic losses for the cotton industry.[7] Addressing these challenges is crucial for sustaining cotton production and ensuring the economic well-being of cotton-producing regions. Consequently, strategies for preventing and controlling Verticillium wilt of cotton, as well as mitigating the impact of soil salinization on the cotton industry, have attracted extensive attention nowadays.

At present, the primary approaches for controlling plant fungal diseases include breeding resistant varieties, crop rotation, and chemical control. Although resistant varieties have demonstrated excellent control efficiency, the lengthy selection and breeding processes present substantial challenges to their widespread implementation. Chemical control, although effective to some extent, is limited by high costs, and poses adverse health and environmental risks.[8] Additionally, crop rotation can lead to increased operational costs and labor requirements. In contrast, biological control has emerged as a promising alternative due to its eco-friendliness and cost-effectiveness.[9] Biological control agents, which include microorganisms, antibiotics, and plant elicitors, were increasingly used to combat plant fungal diseases and have gradually been used in agricultural production.[10] Among these agents, biocontrol microorganisms are widely employed for their ability to induce plant systemic resistance, outcompete pathogenic fungi for nutrients and colonizing space, produce plant hormones, provide nutrients to promote plant growth, and inhibit pathogen infection.[11]

Biocontrol bacteria, particularly *Bacillus* strains such as *B. subtilis*, *B. cereus*, *B. velezensis*, *Bacillus pumilus*, *Bacillus mojavensis*, and *B. halodurans*, play an important role in controlling plant fungal diseases.[12–14] For instance, *B. halotolerans* could effectively inhibit the occurrence of tomato wilt caused by *Fusarium oxysporum f*. sp. *Radicis-lycopersici* and also exhibits strong inhibition of *Alternaria alternata*, *Botrytis cinerea*, *Rhizoctonia bataticola*, and *Phytophthora infestans*.[15] In addition, some *Bacillus* strains not only exhibit salt tolerance but also improve the salt tolerance of plants.[16] It has been reported that *B. halotolerans* strains NMCN1 and LLCG23 possess unique genetic traits that enable them to combat salt stress, making them suitable for use in different bioformulations to enhance crop productivity in saline soils.[17] *B. mojavensis* is widely distributed with biological security and environmental safety, significantly contributing to the biological control of plant fungal diseases. The strain *B. mojavensis* PS-17 has been shown to inhibit the growth and development of pathogenic fungi, exhibiting halotolerance at 5% (w/v) of NaCl and drought tolerance at an osmotic pressure of −2.2 MPa.[18,19] Additionally, *B. mojavensis* BQ-33 serves as a potential biocontrol agent against kiwifruit black spot and other plant diseases caused by fungal pathogens.[14] *B. mojavensis* I4 could help improve wheat's tolerance to salt stress and can also be used as a biofertilizer to enhance plant growth in saline soils and support agriculture practices, which contribute to food security in these challenging global conditions.[20] Moreover, *B. mojavensis* has been found to alleviate salt stress in cotton plants, demonstrating its dual benefits in disease control and stress tolerance. Therefore, *B. mojavensis* presents a promising application prospect in crop disease control.

In this study, to better understand the antagonistic strain KRS009, a series of experiments were performed: the broad-spectrum antifungal activity against seven pathogenic fungi was determined by plate confrontation and fumigation test; the taxonomic features were determined through morphological identification, phylogenetic analysis, and physiological and biochemical characteristics; the effect of KRS009 on the growth and development of *V. dahliae* was studied by microscopic examination; the biocontrol functions against cotton Verticillium wilt and the promotion of plant growth were assessed; and the salt tolerance of KRS009, along with its ability to enhance plant salt tolerance, was evaluated. This work provides a foundation for utilizing strain KRS009 as an effective

biological agent to control Verticillium wilt in hypersaline environments.

2 | MATERIALS AND METHODS

2.1 | Growth of microbes and plants

The strain KRS009 was isolated from the rhizosphere soil of a healthy plant in a pathogen-infested cotton field in Xinjiang Province, China, and has been deposited in the China General Microbiological Culture Collection Center (CGMCC No. 27656). KRS009 was cultured in an LB medium at 28°C. The pathogenic fungi including *V. dahliae*, *F. oxysporum*, *Colletotrichum gloeosporioides*, *Magnaporthe oryzae*, *B. cinerea*, *Colletotrichum falcatum*, and *F. graminearum* were cultured on PDA plates at 25°C. The *Gossypium hirsutum* (Junmian No. 1) cotton seedlings were grown in a greenhouse at 25°C with a 16 h light/8 h darkness photoperiod.

2.2 | Antifungal activity assays in vitro

The effects of KRS009 on the colony growth of seven pathogenic fungi were evaluated through confrontation and fumigation culture assays. For the confrontation culture assays, a fungal bulk with a diameter of 6 mm was inoculated at the center of a PDA plate, while a 10-μL suspension of KRS009 (OD_{600} = 1.0) was dropped 2 cm away from the fungal patch. 10 μL of LB broth dropped at the same distance from the fungal patch served as a control. All plates were cultured in the dark with 25°C for three to seven days. The inhibition rate (%) was calculated using the formula [(C-T)/(C-3)] × 100, where 'C' represents the semidiameter of the fungal colony in the control group, and 'T' represents the semidiameter of the fungal colony in the treatment group. Both the control and treatment groups were performed in triplicate, and the experiment was repeated three times. For the fumigation culture assays, the antifungal activity of the volatile organic compounds (VOCs) released from KRS009 against the seven fungal pathogens was evaluated using two sealed base plate assays.[21] The diameters of the fungal colonies in the control and treatment groups were marked as 'c' and 't', respectively, and the inhibition rate (%) was calculated as [(c-t)/(c-6)] × 100. The culture conditions and experimental replications remained consistent with those described above.

2.3 | Identification and evaluation of basic characteristics of strain KRS009

The cell suspensions of KRS009 were stained using the Gram staining kit (Coolaber, SL7040), including initial dyeing, mordant dyeing, decolorization, and redyeing.[22] A single colony of KRS009 was inoculated in 50 mL of LB, LBGM, and MSgg broth, respectively, shaken at 200 rpm for 24 h at 28°C, and then statically cultured at 28°C for 5 days to observe biofilm formation. The media components of LBGM and MSgg were referenced from previous research.[23]

Phylogenetic analysis was performed to assign the taxonomy of strain KRS009 using multilocus sequence analysis and typing (MLSA-MLST) according to the previously described method with modifications.[24,25] Four housekeeping genes (*rpoB*, *purH*, *gyrB*, and *rpoD*) of strain KRS009 were amplified using the specific primers listed in Table S1 in Supporting Information S2.[26,27] The PCR amplification procedure included initial denaturation at 95°C for 5 min, 33 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 90 s, and a final extension at 72°C for 5 min. The sequences were then aligned with the reference genome of *Bacillus* sp. strains from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), followed by the construction of a phylogenetic tree using MEGA11 and a further customization by TVBOT.

The physiological and biochemical characteristics of KRS009 were assessed based on the methods of Bergey's Manual of Systematic Bacteriology.[28] The 10-μL cell suspension (OD_{600} = 1.0) of KRS009 was inoculated on different functional characteristic plates and incubated at 28°C for two to four days, with *Escherichia coli* DH5α serving as a control. The activity of amylase and protease, along with the utilization of inorganic phosphorus, organic phosphorus, nitrogen, potassium, siderophore production, and indole production were assessed according to the methods described by Wang et al.[21] The saline-alkaline tolerance of KRS009 was evaluated by adding cell suspensions (1% v/v, OD_{600} = 1.0) to an LB medium with varying salt concentrations and pH levels. Temperature tolerance was tested at 25°C, 28°C, 30°C, 37°C, and 42°C. A safety evaluation of the strain KRS009 was conducted by observing the damage of plant cells and a hemolysis experiment in vitro.[29] All assays were repeated for twice, with each treatment comprising three replicates.

2.4 | Microscopic observations

The strain KRS009 was cultured in an LB medium at 28°C for 72 h, after which it was centrifuged to obtain the supernatant with 8000 rpm for 15 min. This supernatant was then filtered using a sterile filter (0.22 μm), and the cell-free fermentation supernatant was harvested. Subsequently, the cell-free fermentation supernatant was co-incubated with the conidial suspension of *V. dahliae* for 24 h, and conidial

germination was observed using a differential interference contrast (DIC) microscope. The hyphal morphology, along with the VOCs released from KRS009 and the 20% cell-free fermentation supernatant, was observed using a scanning electron microscope (SEM). The treatment methods were referenced to the studies of Shan et al.[30] and Wang et al..[21]

2.5 | Biocontrol effect and plant growth promotion assays

The 3-week old cotton seedlings were used to test the biocontrol effect of the strain KRS009 against Verticillium wilt. The conidial suspension with 1×10^7/mL of *V. dahliae* and the KRS009 cell suspension with an OD_{600} of 1.0 were prepared. The biocontrol effect assay included four groups as following: the 'Mock' group, which was treated with water only; the 'KRS009' group, which was irrigated with KRS009 cells' suspension; the 'Vd' group, which was inoculated with the conidial suspension of *V. dahliae*; and the 'KRS009_Vd' group, which was inoculated with the conidial suspension of *V. dahliae* after being treated with the KRS009 cell suspension for 7 days. All treatments were conducted in the same conditions as described before, and the disease phenotypes were observed. The disease index (DI) was calculated using the formula: DI = [Σ(the seedling of every grade × relative grade)/(total seedlings × the most serious grade)] × 100.[31] The relative biomass of *V. dahliae* in the 'KRS009_Vd' group was determined by quantitative PCR (qPCR) as previously described.[32] For the plant growth promotion assays, the plant height, stem diameter, fresh weight, dry weight, the relative content of chlorophyll (the value of Soil and Plant Analyzer Development, SPAD), and nitrogen content in cotton were recorded 30 days after treatment with KRS009 cells' suspension. Each experiment was performed with the same batch of cotton seedlings, with at least 20 cotton seedlings for each treatment, and the experiment was repeated three times.

To detect the expression levels of defense-related genes in cotton seedlings, roots from the 'Mock' and 'KRS009' groups were collected. Total RNA was extracted using an EASYspin plus RNA speed extract kit (Aidlab, Beijing, China), the first-strand cDNA was synthesized with a cDNA Synthesis SuperMix kit (Trans-Gen, Beijing, China), and RT-qPCR was carried out using TransStart Top Green qPCR SuperMix (+DyeII) Kit (Trans-Gen, Beijing, China) according to the manufacturer's instructions. Expression levels of the salicylic acid (SA)- and jasmonic acid (JA)-pathway-related genes were detected, and the *G. hirsutum UBQ7* (*GhUBQ7*) was used as the reference gene for normalization. This assay was repeated twice, with each containing three technical replicates. The results were evaluated using the $2^{-\Delta\Delta CT}$ method as described previously.[33] The primer pairs are listed in Table S1 in Supporting Information S2.

2.6 | Salt tolerance assay of cotton seedlings

To test whether KRS009 can improve salt tolerance in cotton seedlings, we conducted a series of experiments. Firstly, the growth of cotton seedlings under different salt concentration gradients, including 200 mM, 300 mM, 400 mM, 500 mM, and 600 mM NaCl, were assessed to establish a threshold for salt tolerance in cotton seedlings. Secondly, a randomized complete block design was used with two factors at two levels, including non-inoculation and inoculation with strain KRS009, across NaCl concentrations of 0 and 600 mM. This setup resulted in four groups: Mock, KRS009, 600 mM NaCl, and KRS009_600 mM NaCl. Each treatment was replicated three times. A 2-mm soil sieve was used to filter the nutrient soil. The ratio of NaCl solution to soil is 9:8 (V/W, mL/g).

Four groups were prepared as follows: Mock: 900 mL of sterile ddH_2O; KRS009: cultured in an LB medium and incubated by shaking (200 rpm) for 72 h at 28°C, followed by cell collection by centrifugation and resuspension in 900 mL of sterile ddH_2O until reaching a final OD_{600} of 0.5; 600 mM NaCl: a 900-mL NaCl solution with a concentration of 600 mM; KRS009_600 mM NaCl: KRS009 cells recovered by centrifugation and resuspended in the 900-mL NaCl solution (600 mM) until reaching a final OD_{600} of 0.5.[34] Plants subjected to treatments without salt stress were irrigated with sterile ddH_2O. The pots were individually weighed daily using the method of specific gravity, and the saline solution (600 mM NaCl) was applied to maintain a consistent salt concentration across all pots. Each group was conducted with at least three replicates, and this assay was performed three times.

For the detection of expression levels of salt tolerance-related genes, including *GhARF*, *GhWRKY41*, *GhMYB1*, *GhMAPK*, *GhbHLH*, *GhDBP2*, *GhC2H2*, *GhCBL3*, *GhCBL4*, and *GhSOS4*,[35–39] in cotton seedlings, the roots of cotton seedlings from four distinct groups were collected. The RNA extraction, cDNA synthesis, and RT-qPCR detection were conducted as above. The primer pairs are listed in Table S1 in Supporting Information S2.

2.7 | Statistical analyses

The data presented in this study were shown as mean ± SD ($n = 3$), and an unpaired Student's *t*-test was performed to determine statistical significance. Statistical analyses were performed using SPSS 17.0

(IBM, Chicago, USA). The means were compared by analysis of variance (ANOVA), and mean differences were evaluated using Duncan's multiple range tests. A significance level of $p < 0.01$ was considered statistical significance.

3 | RESULTS

3.1 | KRS009 has broad-spectrum inhibitory activity in vitro and was identified as *B. mojavensis*

In order to determine the spectrum of inhibitory activity of KRS009, confrontation tests and covering fumigation treatments were conducted. In the confrontation test, strain KRS009 exhibited a strong inhibitory effect on the mycelial growth of seven pathogens (Figure 1A). The fermented broth of strain KRS009 significantly inhibited the growth of *V. dahliae*, *B. cinerea*, and *M. oryzae*, with suppression rates of 90.15%, 78.97%, and 97.42%, respectively. Additionally, strain KRS009 showed inhibitory effects on *C. gloeosporioides*, *C. falcatum*, *F. graminearum*, and *F. oxysporum*, with inhibition rates of 44.20%, 43.58%, 32.86%, and 67.19%, respectively (Figure 1B,C). These results suggest that strain KRS009 may possess the ability to secrete antifungal metabolites that exert inhibitory activity. Furthermore, to distinguish the antifungal effects of KRS009 cells and their secondary metabolites, cell suspension (CS) and fermentation supernatant (FS, also referred to as cell-free supernatant), respectively, were collected to evaluate their inhibitory functions. The CS and FS of KRS009 exhibited varying degrees of inhibitory effects on different fungi. Especially in the cell suspension of KRS009, whether at 10% or 20% (v/v), the inhibitory rate exceeded 50% (Figure S1, S2 in Supporting Information S1). Further investigation was conducted to study whether VOCs inhibit the growth of pathogenic fungi. Through the covering fumigation treatment, the inhibitory effects of VOCs on the growth of seven pathogens were evaluated. The results indicated that KRS009 exhibited varying degrees of inhibitory effects on these seven pathogenic fungi (Figure 1D). Notably, the colony growth of *B. cinerea* was significantly inhibited by strain KRS009, achieving a suppression rate of 88.32%. Additionally, the inhibition rates against *V. dahliae, C. gloeosporioides*, *C. falcatum*, *F. graminearum*, *M. oryzae*, and *F. oxysporum* were 29.85%, 27.27%, 21.92%, 28.65%, 38.79%, and 35.82%, respectively (Figure 1E,F). Taken together, these results demonstrate that strain KRS009 possesses a broad-spectrum inhibitory effect on plant pathogens.

Morphologically, the colony of strain KRS009 appeared oyster white and opaque, forming rough, wrinkled circular colonies on the LB medium (Figure S3A in Supporting Information S1). SEM observation indicated that KRS009 is a short rod-shaped bacterium, with length ranging from 1.5 to 2.5 μm and widths from 0.5 to 0.8 μm (Figure S3B in Supporting Information S1). Gram staining indicated that KRS009 is a gram-positive, blue-purple colored, short coryneform bacterium (Figure S3C in Supporting Information S1). The strain KRS009 demonstrated the ability to form biofilms, with *E. coli* DH5α serving as the negative control (Figure S3D and S4 in Supporting Information S1). Phylogenetic analysis confirmed that strain KRS009 belongs to the clade I group of *B. mojavensis*, based on multilocus sequence analysis and typing (MLSA-MLST) involving tandem loci such as *rpoB*, *purH*, *rpoD*, and gyrB (Figure S3E in Supporting Information S1).[12]

3.2 | Physiological and biochemical analysis of KRS009

The physiological and biochemical characteristics of KRS009 are summarized in Table S2 in Supporting Information S2. In this study, the strain KRS009 exhibited a variety of enzymatic activities, including amylase, protease, oxidase, catalase, and gelatinase (Figure S5A in Supporting Information S1). These activities may play crucial roles in enhancing the degradation of the cell wall of pathogens, and potentially providing accessible energy sources for the bacterium. Additionally, KRS009 demonstrated a strong capacity to solubilize potassium, indicating its potential as a plant growth-promoting agent (Figure S6A in Supporting Information S1). Furthermore, as an aerobic bacterium, KRS009 showed the capacity to reduce nitrate (Figure S6B in Supporting Information S1). Negative results were observed for the phenylalanine deaminase test, inorganic phosphate dissolution, organophosphate dissolution, nitrogen fixation, siderophore detection test, indole test, methyl red test, citrate utilization, hydrogen sulfide test, and glucose utilization (Figure S5A and S6A-B in Supporting Information S1).

Safety assessment is crucial for the potential application of biocontrol microorganisms. Pathogenicity and hemolysis tests were performed to evaluate the safety of strain KRS009. Trypan blue staining revealed no necrosis in cotton roots inoculated with strain KRS009, indicating no adverse effects compared to the control. In contrast, roots soaked in *Ralstonia solanacearum* fermentation broth exhibited blue dye accumulation (Figure S5B in Supporting Information S1). To ensure human safety and health, the hemolytic test on Columbia and Mueller–Hinton blood plates indicated the absence of α-hemolysin in KRS009, as evidenced by the lack of a hemolytic ring around the colony,

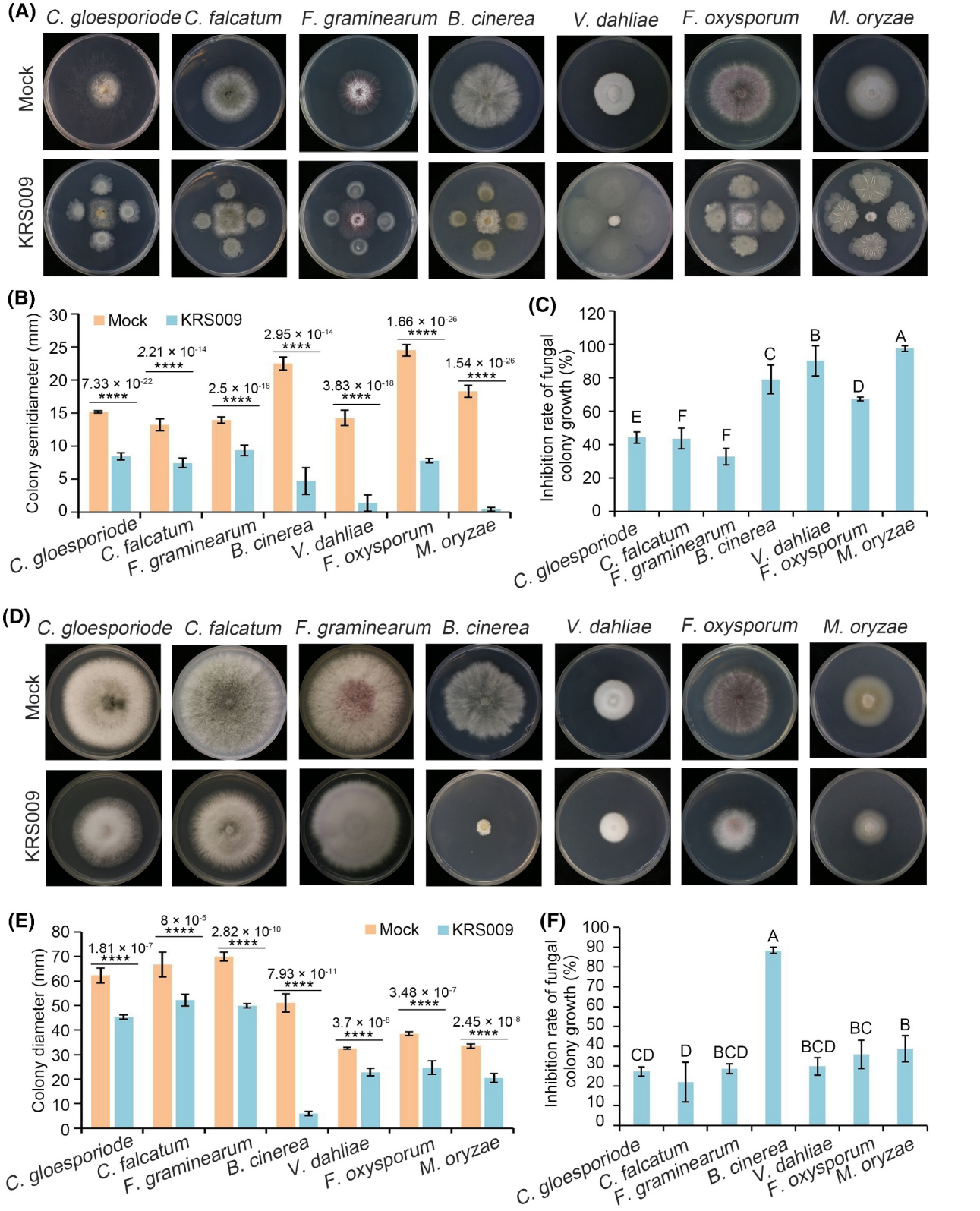

FIGURE 1 **The strain KRS009 exhibits broad-spectrum inhibitory activities against plant pathogenic fungi**. (A) The antifungal activity of strain KRS009 against seven plant pathogenic fungi was determined using plate antagonistic culture method (B, C) The semidiameter of the fungal colonies and the inhibition rates of their growth were measured through the confrontation culture assay (D) The antifungal activity of volatile organic compounds (VOCs) was determined by the covering fumigation method (E, F) The diameter of the fungal colonies and their corresponding inhibition rates were measured through the covering fumigation. Error bars represent standard errors. ****: significant differences at $p < 0.0001$ according to an unpaired Student's *t*-test. Based on one-way analysis of variance (ANOVA), the letters (A to F) in the above column indicate significant differences at the $p \leq 0.01$ level. *Verticillium dahliae* (Vd); *Colletotrichum falcatum* (Cf); *Colletotrichum gloeosporioides* (Cg); *Fusarium graminearum* (Fg); *F. oxysporum* (Fo); *Magnaporthe oryzae* (Mo); and *Botrytis cinerea* (Bc).

confirming that KRS009 does not break down red blood cells (Figure S5C in Supporting Information S1). These results affirm the safety and innocuous nature of strain KRS009.

Given the increasing focus on crop cultivation in high-temperature and saline-alkali areas,[40] we evaluated the resistance of strain KRS009 to both high temperatures and saline-alkali conditions. Culturing KRS009 at various temperatures (25°C, 28°C, 30°C, 37°C, and 40°C) revealed its significant heat resistance relative to the control group, with optimal growth at 28°C, identified as the most suitable culture temperature (Figure S7A,B in Supporting Information S1). Similarly, culturing KRS009 at different pH levels (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0) demonstrated alkaline resistance compared to the control group, with optimal growth at pH 7.0, which was determined to be the most suitable culture pH (Figure S7C,D in Supporting Information S1).

3.3 | The strain KRS009 inhibited the morphological development of *V. dahliae*

Conidial germination was evaluated as a key factor influencing the outbreak of plant fungal diseases. Microscopic observations revealed that conidia treated with KRS009 culture filtrate exhibited reduced or no germination compared to the control group, with a decrease in spore concentration as the treatment period increased (Figure 2A,B).

Inoculating *V. dahliae* on a PDA plate containing 20% extracellular metabolites of KRS009 or VOCs produced by KRS009 resulted in significant growth inhibition (Figure 2C,D), with suppression rates of 35.08% and 67.35%, respectively (Figures S8A and 8B in Supporting Information S1). Moreover, SEM observations showed that the morphology of *V. dahliae* mycelia treated with KRS009 showed rough folds, disordered growth, breakage, and hollow formation, whereas the control group exhibited smooth, intact hyphae with normal growth (Figure 2E,F). The results of stereomicroscopic observations further supported above findings, revealing disordered, expanded, and broken mycelia in the treatment group compared to the control (Figures S8C and 8D in Supporting Information S1).

3.4 | The strain KRS009 can protect cotton from *Verticillium dahliae*

We further investigated whether strain KRS009 could prevent the onset of cotton Verticillium wilt in pot assays. The cotton group infected solely with the pathogenic fungus *V. dahliae* exhibited disease symptoms, including slow growth, yellowing leaves, and defoliation. In contrast, cotton treated with KRS009 exhibited robust growth and enhanced resistance to Verticillium wilt compared to control plants (Figure 3A and Figure S9 in Supporting Information S1). Moreover, the efficacy of KRS009 was comparable to that of the chemical fungicide tebuconazole and other *Bacillus* strains, including *B. amyloliquefaciens* KRS005[41] and *B. subtilis* KRS015 (Figure S9 in Supporting Information S1).[31] Longitudinal cutting of the cotton stem revealed browning and wilting in the vascular bundles of cotton from the group infected solely with *V. dahliae*, whereas no such symptoms were observed in the treatment group (Figure 3A). The disease index for the group infected with the suspension of *V. dahliae* conidia alone was 90.16%, with disease grades at levels G1 (6.41%), G2 (16.68%), and G3 (76.90%). In comparison, the group treated with the suspension of *V. dahliae* conidia following application of KRS009 fermentation broth exhibited a disease index of 1.60%, with disease grades at levels G0 (95.19%) and G2 (4.81%) (Figure 3B,C). Fungal biomass analysis using quantitative PCR (qPCR) indicated a significant reduction in fungal growth when treated with KRS009 fermentation broth compared to the control (*V. dahliae*) (Figure 3D). These findings confirm that strain KRS009 can protect cotton from *V. dahliae*, as validated in greenhouse experiments.

Previous studies have demonstrated that biocontrol microorganisms can induce an immune response in plants by secreting secondary metabolites that enhance their ability to resist biotic stress, such as pathogenic fungi. To investigate whether KRS009 could trigger the plant defense response, the expression levels of the related marker gene in the salicylic acid (SA)- and jasmonic acid (JA)-signaling pathways were examined using reverse transcription quantitative PCR (RT-qPCR). Cotton plants treated with KRS009 fermentation broth for two days showed a significant induction of marker genes in the SA- (*GhEDS1*, *GhICS, GhNPR1*, *GhNDR1*, *WRKY7*, *GhPR1*, and *GhPR5*) and JA- (*GhAOS*, *GhJAZ*, *GhAOCS*, *GhLOX1*, and *GhOPR3*) signaling pathways compared to the water-treated control cotton (Figure 3E,F). These results suggest that KRS009, effectively inhibits the occurrence of Verticillium wilt, potentially by stimulating the plant immune response associated with the SA- and JA-signaling pathways.

Plant growth-promoting rhizobacteria (PGPR) represent a diverse group of plant-associated microorganisms that colonize plant roots, interact with plants, stimulate growth, and positively influence plant metabolism. *B. mojavensis* has been identified as a typical PGPR.[42] The impact of *B. mojavensis* KRS009 (OD_{600} = 1.0) on cotton growth was investigated (Figure S10A in Supporting Information S1). Treatment

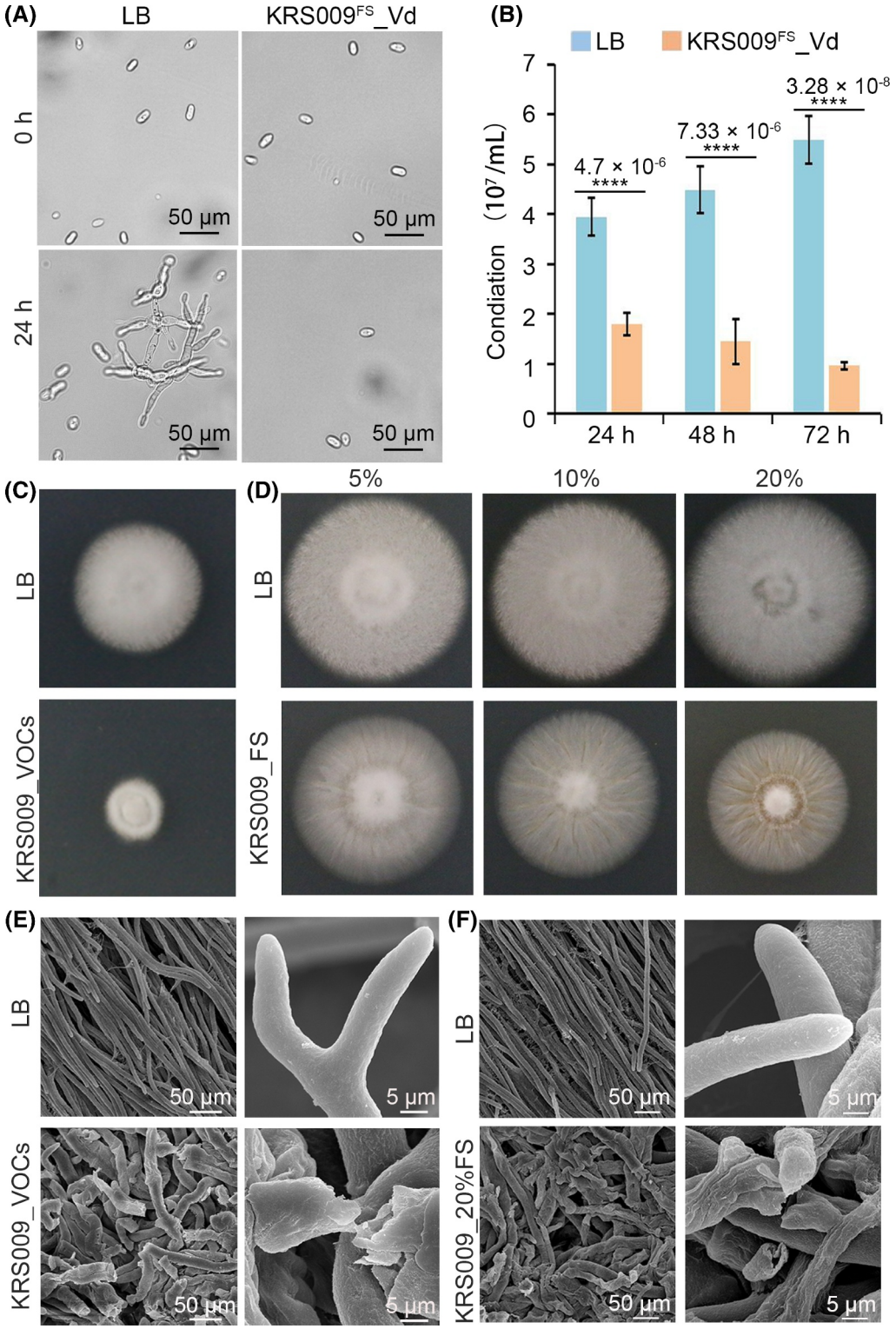

FIGURE 2 **The Strain KRS009 influences on the growth and development of *V. dahliae*.** (A) The spore germination of *V. dahliae* in the presence of KRS009 fermentation broth was observed using a differential interference contrast microscope (DIC). The spores incubated in LB broth were set as the control (B) The amount of sporulation at different time points was counted by incubating KRS009 fermentation supernatant (KRS009-FS) with the *V. dahliae* spore solution. The **** indicates statistical difference with $p \leq 0.0001$ compared to the control group (C) The inhibitory activity of covering fumigation of KRS009 against *V. dahliae* grown on potato dextrose agar (PDA) plates was assessed, with covering fumigation of a Luria-Bertani (LB) solid medium against *V. dahliae* as control group (D) The antifungal activity of different concentrations of KRS009 fermentation supernatant (KRS009-FS) against *V. dahliae* on PDA plates was evaluated, with different concentrations of an LB solid medium used as controls (E, F) A scanning electron microscope (SEM) was used to examine hyphal morphology defects from the edges and superficial state, as well as from the clear outline and internal cellular state of the inhibitory zone of *V. dahliae*, due to the secondary metabolites and VOCs of strain KRS009. The ruler at the bottom right of the image indicates the actual size.

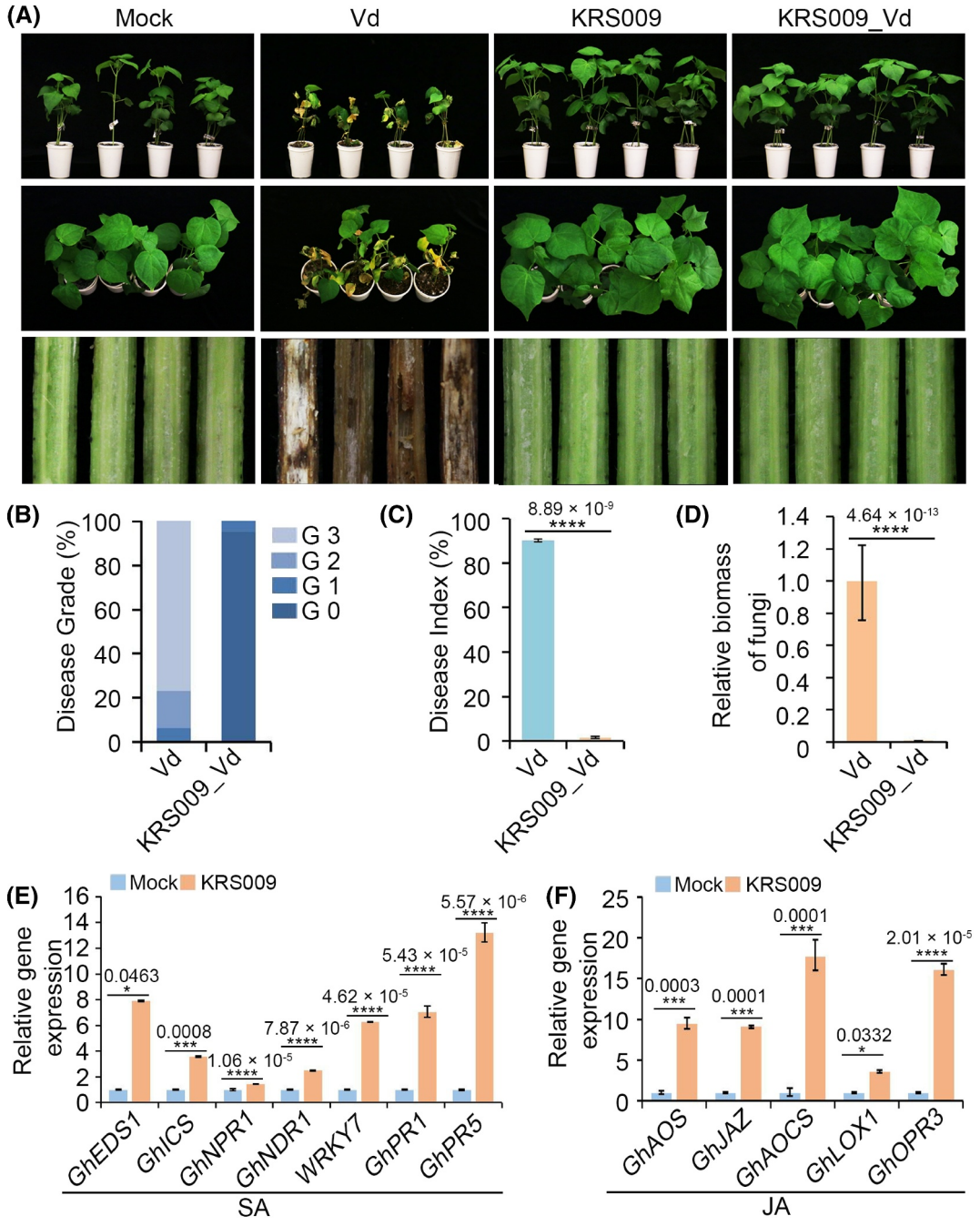

FIGURE 3 **The efficacy of KRS009 in controlling Verticillium wilt in cotton**. (A) The cotton plant phenotypes after the treatments with uninoculated control (Mock), inoculation of *V. dahliae* (Vd), inoculation of *Bacillus mojavensis* KRS009 (KRS009), and inoculation of *B. mojavensis* KRS009 followed by *V. dahliae* 7 days later (KRS009_Vd) were collected 21 days after *V. dahliae* infection, respectively (B) The statistical analysis of the disease grade of Verticillium wilt in cotton (C) The disease index of Verticillium wilt on cotton was evaluated 21 days after *V. dahliae* infection (D) Fungal biomass of *V. dahliae* on cotton roots was determined by quantitative PCR (qPCR) (E, F) The relative expression levels of defense-related genes involved salicylic acid (SA) and jasmonic acid (JA) pathways. Error bars represent standard errors. *, **, ***, and **** denote significant differences at $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively, between the treatment and control groups according to unpaired Student's *t*-test.

with KRS009 markedly increased plant height, stem diameter, fresh weight, and dry weight of cotton compared to the control (Figure S10B in Supporting Information S1). Furthermore, the contents of chlorophyll and nitrogen in leaves of cotton treated with KRS009 fermentation broth were increased through a chlorophyll analyzer (Figure S10C in Supporting Information S1). In conclusion, the KRS009

fermentation broth effectively promotes the growth of cotton plants.

3.5 | KRS009 has the ability to tolerate salt

Observations of strain KRS009 growth in an LB medium with varying sodium chloride concentrations revealed that KRS009 exhibited enhanced salt tolerance compared to the DH5α control group (Figure 4A,B). After 5 days of static culture, biofilm formation was observed in the KRS009 culture. The formation of biofilm was positively correlated with the increase of NaCl concentration (Figure 4C). The verification of salt tolerance in KRS009 was further confirmed by culturing on Luria-Bertani (LB) plates with different salt concentrations (Figure 4D). Moreover, the expression levels of exopolysaccharides (EPS)-related genes[43] showed that *eps-J* and *eps-L* were upregulated during biofilm formation under salt stress (Figure S11 in Supporting Information S1). These results indicate that KRS009 not only possesses salt tolerance but also has the capability to form the biofilm under relatively high salt stress.

3.6 | KRS009 can improve plant salt tolerance

In order to study the role of KRS009 in cotton adaptation to salt stress, a salt tolerance test was conducted. Six groups were established, including Mock, 200 mM, 300 mM, 400 mM, 500 mM, and 600 mM NaCl. The emergence of cotton was monitored on the seventh, 14th, and 21st days, with the emergence rate calculated based on the appearance of two cotyledons as the standard. The results indicated that NaCl treatment resulted in a decreasing trend in emergence rates of seeds and an increasing trend in emergence time, and the emergence rate in 600 mM NaCl was lower than other treatments with lower concentrations (Figure 5A and Figure S12A in Supporting Information S1). To investigate the effects of NaCl on cotton plants, growth parameters from the above five different salt stress treatment groups were recorded and compared with the control. The results indicate that at an NaCl concentration of 300 mM, there is an observed increase in SPAD values, plant height, nitrogen content, fresh weight, and dry weight in cotton. In contrast, at an NaCl concentration of 600 mM, these parameters show a

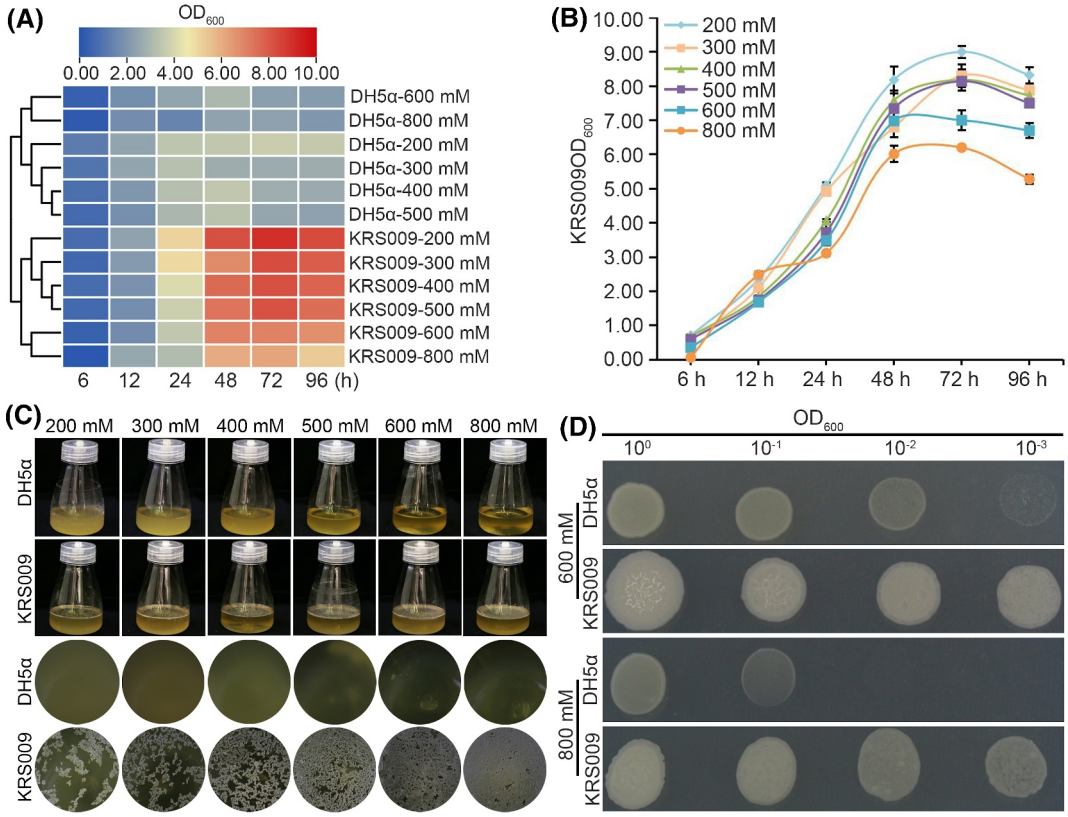

FIGURE 4 **KRS009 possesses the ability to tolerate salt**. (A) The growth of KRS009 was observed at different salt concentrations, with *Escherichia coli* DH5α serving as the negative control (B) KRS009 was cultured at different salt concentrations to evaluate its growth (C) The biofilm formation of KRS009 was observed by culturing in a Luria-Bertani (LB) medium with different salt concentrations, with *E. coli* DH5α as the negative control (D) The salt tolerance of KRS009 was evaluated by culturing on Luria-Bertani (LB) plates with different salt concentrations, with *E. coli* DH5α cultured as the negative control.

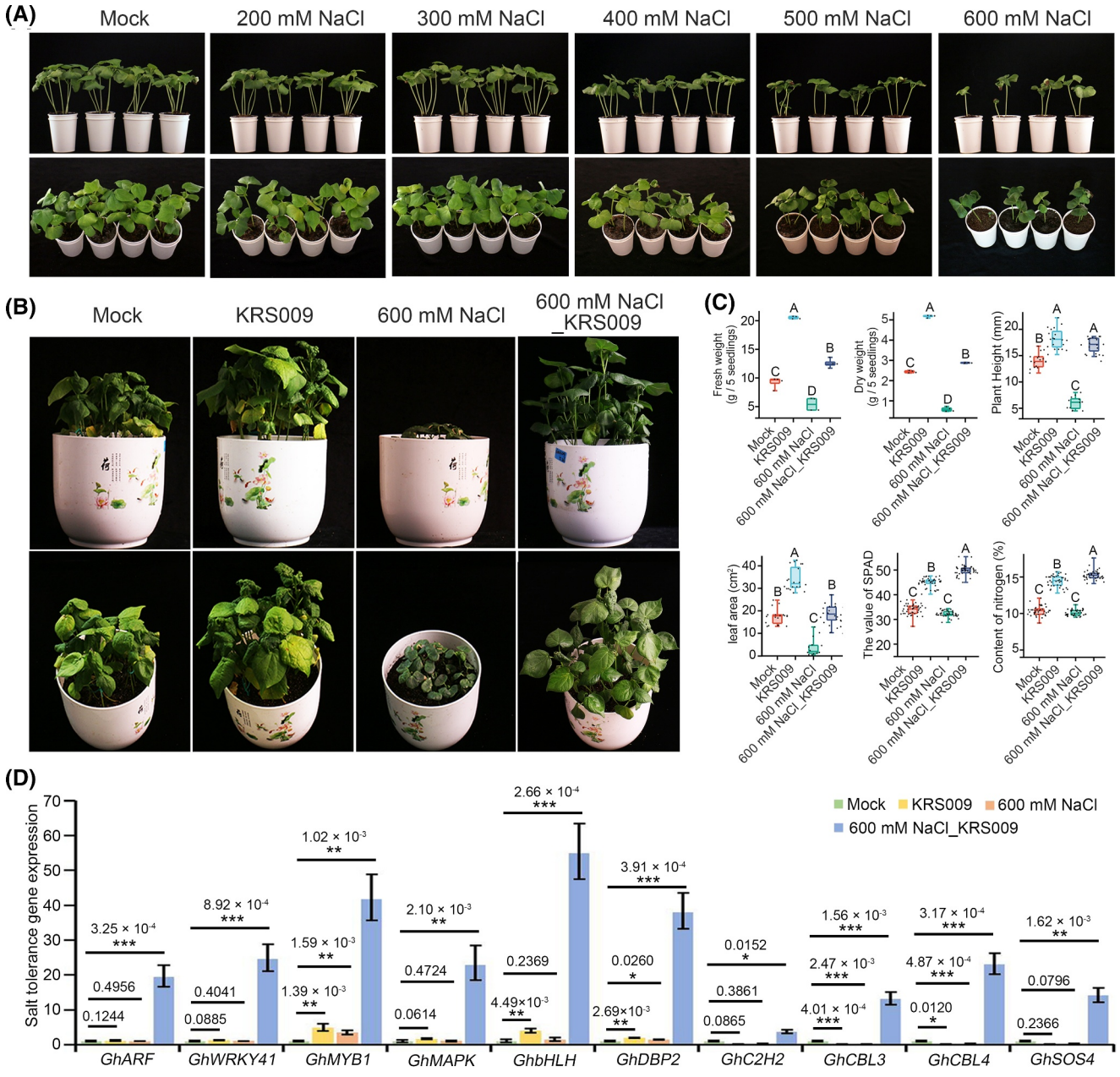

FIGURE 5 KRS009 improves the tolerance of cotton to salt stress. (A) The phenotype of cotton under different NaCl concentrations (B) Determination of the ability of KRS009 to relieve salt stress in cotton. Four groups are set as following: KRS009, 600 mM NaCl, KRS009 mixed with 600 mM NaCl (600 mM NaCl_ KRS009), and control group treated with water only (C) The fresh weight, dry weight, plant height, leaf area, value of SPAD and content of nitrogen in cotton seedlings were measured in the above four groups (D) Relative expression of salt tolerance genes in cotton roots treated with KRS009 detected by qPCR. Each treatment value is presented as the mean of three replications ($n = 3$) with the standard error (SE). Error bars represent standard errors. *, **, ***, and **** stand for the significant differences at $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively, between the treatment and control groups according to unpaired Student's *t*-test.

significant decline (Figure S12B–F in Supporting Information S1). These findings indicate that an optimal salt concentration can promote the growth of cotton plants, whereas elevated salt concentration can impede healthy development, establishing 600 mM NaCl as the critical threshold for salinity tolerance in cotton.

Subsequently, several growth index of cotton seedlings treated with KRS009 under salt stress was investigated in a potted plant experiment. In the "600 mM NaCl " group, cotton emergence was much lower than in the "Mock" group, with slow seedling development and dwarfed plants (Figure 5B). Parameters such as cotton fresh weight, dry weight, plant height, leaf area, nitrogen, and chlorophyll levels were all significantly diminished in the "600 mM NaCl " group compared to the "Mock" group (Figure 5C). Conversely,

"KRS009" demonstrated a strong capacity to enhance growth, leading to increases in the aforementioned diameters of cotton compared to the control (Figure 5B,C). Notably, the cotton plants in the "600 mM_KRS009" group exhibited superior growth compared to those in the "600 mM NaCl" group, even surpassing the control group, with elevated fresh weight, dry weight, plant height, leaf area, chlorophyll, and nitrogen contents (Figure 5B,C). These results demonstrate that strain KRS009 not only alleviates the salt stress but also promotes the growth of cotton under such conditions.

Each stress factor triggers a complex cellular and molecular network in crop plants, which utilize a complex antioxidative defense system to repair or prevent damage through inducing stress-related genes.[35] To investigate whether KRS009 can induce plant defense responses, RT-qPCR was conducted to assess the expression levels of salt tolerance-related genes. Transcription factors such as *WRKY*, *bHLH*, *MYB*, and *ARF* are known to respond to salt stress.[36] The expression levels of these genes were all upregulated in the KRS009 treatment group under salt stress. Notably, the *C2H2* zinc finger protein, which belongs to the largest transcription factor family in plants, was identified solely in the forward library. The expression levels of *GhARF*, *GhWRKY41*, and *GhC2H2* genes in the KRS009 and the salt stress-treated group were significantly upregulated than the salt stress treatment only (Figure 5D). Moreover, treatment with KRS009 at 600 mM salinity in cotton resulted in a considerable upregulation of the DREB/CBF subfamily genes *GhMAPK*, *GhSOS4*, *GhCBL3*, *GhCBL4*, and *GhDBP2* (Figure 5D).[44] Together, these findings showed that KRS009 had the ability to enhance the salt tolerance of cotton.

4 | DISCUSSION

The rhizosphere is the region surrounding plant roots where microbial activity is maximized. Beneficial and harmful activities of microorganisms in the rhizosphere play a critical role in influencing plant growth and development.[45] Microbial community in the rhizosphere is dominantly composed of bacteria, followed by fungi, protozoa, Actinomycetes, etc.[46] Mutualistic rhizospheric bacteria that improve plant growth and health are referred to as PGPR. These bacteria are essential due to their multifaceted contributions to plant well-being,[47] For instance, *B. subtilis* YB-15 has been identified as a promising biocontrol agent against Fusarium crown rot and a promoter of wheat growth. Reduced Fusarium crown rot can be attributed to direct antagonism by the production of β-1,3-glucanase, amylase, protease, and cellulase, along with the capacity of *B. subtilis* YB-15 to induce defense-related enzyme activities of wheat seedlings, both independently and in seedlings infected with *F. pseudograminearum*.[48] In this study, we isolated the strain KRS009 from the rhizosphere soil of healthy plants in a cotton field infested with pathogens. Through MLSA-MLST analysis of tandem loci of *rpoB*, *purH*, *rpoD*, and *gyrB* and morphological identification, the strain KRS009 was identified as gram-positive *B. mojavensis* (Figure S3C,E in Supporting Information S1).

PGPRs are recognized for their ability to promote plant growth and improve nutrition, either directly or indirectly. The direct facilitation of plant growth by PGPRs includes the supplementation of essential nutrients such as nitrogen, phosphate, zinc, potassium, and iron, along with the production of phytohormones.[49] Additionally, PGPRs synthesize a variety of enzymes and metabolites, including proteases, amylases, and cellulases, which can inhibit plant pathogens and promote plant growth.[50] The evaluation results for physiological and biochemical characteristics of the strain KRS009 revealed that *B. mojavensis* KRS009, as a PGPR, possesses the ability to solubilize potassium and exhibits enzyme activities such as those of amylase and protease (Figure S5), indicating its potential to inhibit plant pathogens and promote plant growth.

B. mojavensis KRS009 exhibits a broad-spectrum antifungal effect against various fungal species, including *V. dahliae*, *B. cinerea*, *M. oryzae*, *C. gloeosporioides*, *C. falcatum, F. graminearum*, and *F. oxysporum* (Figure 1A,D). Recent reports have also indicated that *B. mojavensis* demonstrates antagonistic properties against both plant and human pathogens, particularly concerning fungal diseases. Additionally, KRS009 was observed to be nontoxic with no phytotoxic effects on cotton buds (Figure S5B in Supporting Information S1). It also did not induce hemolysis in red blood cells based on the hemolysis test (Figure S5C in Supporting Information S1). Therefore, our study characterizes *B. mojavensis* as safe for both plants and humans. The nontoxicity and safety of these strains are important prerequisites for the further development of potential biocontrol microbial resources or biofertilizers.

Previous studies have reported that salinity stress enhances biofilm production in some rhizobacteria.[51] The increase in biofilm production in *Bacillus* spp. under salinity stress is possibly attributed to the increased levels of exopolysaccharides at elevated concentrations of salt.[52] Exopolysaccharides (EPS) play a critical role in mechanically stabilizing the three-dimensional structure of biofilm architecture[53] and in protecting biofilm-forming cells from various environmental stresses. Similarly, the biofilm formation of *B. mojavensis* KRS009 significantly increased at elevated NaCl concentrations (Figure 4C). Biofilm formation is a selective survival strategy for microbial cells in

unfavorable environments[51] and is an important characteristic for better colonization in the rhizosphere and roots,[13] thus prompting increased focuses on the inoculation of biofilm-forming microorganisms. *B. mojavensis* D50's biofilms can affect its colonization, and are used to prevent and treat the fungal plant pathogen *B. cinerea*.[54] Here, the expression levels of EPS-related genes showed that *eps-J* and *eps-L* were upregulated during biofilm formation of KRS009 under salt stress (Figure S11 in Supporting Information S1). Furthermore, biofilm production in salt-tolerant PGPR induced by high salt levels enhances root colonization, alleviates salt stress, and thus improves plant growth.[55] Interestingly, an important link has been found between the exopolysaccharide-producing potential of PGPR and the amelioration of salt stress. Although we did not observe biofilm formation and EPS production on the surface of cotton roots, the observed enhancements in plant growth parameters may be attributed to increased biofilm production by the respective strains under higher salinity.

In response to various environmental stresses, plants initiate multiple perception and signaling pathways, which may interact at different steps. For example, the *MAPK* cascade is essential for various signaling pathways under both biotic and abiotic stresses. Plant subjected to both cold and salt stresses exhibit crosstalks between cold- and salt-tolerance related pathways.[56] Several recent reports also emphasized the importance of transcription factors in mediating responses to abiotic stresses. Different families of transcription factors, such as *WRKY*-type transcription factor families, play diverse roles in plant development and stress regulation.[57] It is well-known that interaction networks during salt stress involve pathways such as those involving salt overly sensitive (SOS), mitogen-activated protein kinase (MAPK), calcineurin B-like proteins (CBLs), and DRE-binding protein 2 (DBP2). The SOS and MAPK pathways play key roles in the salt stress response.[37,38] Previous research has found that *SOS4*, encoding a pyridoxal kinase, is involved in the biosynthesis of pyridoxal-5-phosphate in *Arabidopsis*. Pyridoxal-5-phosphate was supposed to regulate Na^+/K^+ homeostasis by regulating the activities of ion transporters.[58] In this study, KRS009 was found to be instrumental in regulating the expression of plant salt-tolerant genes *WRKY*, *bHLH*, *MYB*, *ARF*, *SOS*, *MAPK*, *CBLs*, and *DBP2* under salt stress, thus providing necessary information for the functional characterization of various salt-tolerant genes in plants under salt stress. These results showed that overexpression of the salt stress response transcription factor in cotton treated with KRS009 could improve the salt tolerance of cotton and increase its yield under salt stress (Figures 5B,D). We measured not only the salt tolerance of strain KRS009 but also the optimal temperature and pH for its growth conditions. These results indicate that strain KRS009 exhibits not only salt tolerance (Figures 4A,B and D) but also resistance to high temperatures and alkaline conditions (Figure S8 in Supporting Information S1), thus establishing a solid foundation for future research on the influence of strain KRS009 on plant growth and development under high-temperature stress and in alkaline environments.

5 | CONCLUSION

In conclusion, *B. mojavensis* KRS009, isolated from the rhizosphere soil of a healthy plant in a pathogen-infested cotton field, was identified through morphological, molecular, and physio-biochemical characteristics. This strain exhibits a broad spectrum of antifungal activity against various fungal pathogens and is safe for plants, animals, and humans. Additionally, *B. mojavensis* KRS009 also demonstrates plant growth-promoting traits in vitro, along with characteristics of salt and alkali resistance, thriving at high temperature and exhibiting biofilm formation. Furthermore, KRS009 effectively mitigates the occurrence of Verticillium wilt in cotton. Additionally, KRS009 strain can also trigger a wide plant immune response, enhancing salt tolerance in cotton plants, which can be improved under salt stress. In summary, KRS009 shows promise as a valuable resource for biocontrol agents and biofertilizers in saline-alkali areas.

AUTHOR CONTRIBUTIONS

Fuhua Zhao: Investigation, data curation, formal analysis, writing-original draft. **Dan Wang**: Investigation, conceptualization, methodology, visualization, writing-original draft. **Huizi Liu**: Investigation, data curation, formal analysis. **Yujia Shan**: Investigation, data curation, formal analysis. **Hongyue Qi**: Investigation, data curation, formal analysis. **Jieyin Chen**: Funding acquisition, conceptualization, resources, supervision, project administration, writing-review and editing. **Didier Lesueur**: Writing-review and editing. **Dongfei Han**: Funding acquisition, conceptualization, supervision, methodology, writing-review and editing. **Xiaojun Zhang**: Writing-review and editing. **Dandan Zhang**: Funding acquisition, conceptualization, resources, supervision, project administration, writing-review and editing.

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CONFLICT OF INTEREST STATEMENT

All authors read and approved the final manuscript. All authors declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

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