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Efficacy evaluation and mechanism of *Bacillus subtilis* EBS03 against cotton Verticillium wilt

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Abstract

Background: In our previous study, a strain EBS03 with good biocontrol potential was screened out of 48 strains of cotton endophyte *Bacillus subtilis* by evaluating the controlling effect against cotton Verticillium wilt. However, its mechanism for controlling Verticillium wilt remains unclear. The objective of this study was to further clarify its controlling effect and mechanism against cotton Verticillium wilt.

Results: The results of confrontation culture test and double buckle culture test showed that the inhibitory effects of EBS03 volatile and nonvolatile metabolite on mycelium growth of *Verticillium dahliae* were 70.03% and 59.00%, respectively; the inhibitory effects of sporulation and microsclerotia germination were 47.16% and 70.06%, respectively. In the greenhouse test, the EBS03 fermentation broth root irrigation had the highest controlling effect at 87.11% on cotton Verticillium wilt, and significantly promoted the growth of cotton seedlings. In the field experiment, the controlling effect of EBS03 fermentation broth to cotton Verticillium wilt was 42.54% at 60 days after cotton sowing, and the boll number per plant and boll weight in EBS03 fermentation broth seed soaking, root irrigation, and spraying treatments significantly increased by 19.48% and 7.42%, 30.90% and 2.62%, 15.99% and 9.20%, respectively. Furthermore, EBS03 improved the resistance of cotton leaves against the infection of *V. dahliae*, and induced the outbreak of reactive oxygen species and accumulation of callose. In addition, the results of real time fluorescent quantitative polymerase chain reaction (RT-qPCR) detection showed that EBS03 significantly induced upregulation expression level of defense-related genes *PAL*, *POD*, *PPO*, and *PR10* in cotton leaves, enhanced cotton plant resistance to *V. dahliae*, and inhibited colonization level of this fungal pathogen in cotton.

Conclusion: *Bacillus subtilis* EBS03 has a good biological defense capability, which can inhibit the growth and colonization level of *V. dahliae*, and activate the resistance of cotton to Verticillium wilt, thus increase cotton yield.

Keywords: Endophytic bacteria, *Bacillus subtilis*, Cotton Verticillium wilt, Control mechanism, Induced resistance

Introduction

As a natural fiber crop, cotton is an important economic crop in the world. Cotton Verticillium wilt, mainly caused by *Verticillium dahliae* Kleb., is a widespread disease that occurs in most cotton-producing areas (Fradin and Thomma 2010; Chi et al. 2021). Due to the stable dormant structure of microsclerotia, this fungus can survive in the soil for more than ten years, and the pathogen population is rich in genetic diversity so the pathogenicity is

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prone to variability (Zhang et al. 2022). Therefore, cotton Verticillium wilt has not been effectively controlled, known as "cotton cancer". At present, traditional methods such as crop rotation, selection of disease-resistant varieties, and chemical controlling are mainly used for the prevention and control of cotton Verticillium wilt in the world, but the prevention and controlling effect is still unsatisfactory (Zhao et al. 2017; Zhang et al. 2021).

With the rise of green agriculture, comprehensive controlling measures based on biological controlling have been paid more and more attention, which is very important in the prevention and treatment of plant diseases (Acharya et al. 2020; Ingram et al. 2020; Mitra et al. 2022). Most of all, it is a research hotspot to screen antagonistic microorganisms from soil and cotton plant to control this disease (Berg et al. 2010; Bubici et al. 2013). Generally, the antagonistic microorganisms used to control diseases include bacteria, fungi, actinomycetes, and so on. The main strategy is to induce plant systemic resistance to inhibit pathogen infection, and then promote plant growth by producing hormones and providing nutrients (Varo et al. 2016; Sehrawat et al. 2022). As an important group of biocontrol microorganisms, bacteria played an important role in controlling of this disease, especially *Bacillus* spp. There were many application cases, such as *B. subtilis* (Zhao et al. 2022), *B. cereus* (Wang et al. 2016), *B. pumilus* (Li et al. 2018), *B. licheniformis* (Gutiérrez-Mañero et al. 2001). *B. subtilis* was widely used to prevent and control plant diseases because of its strong adaptability and good antibacterial activity in the soil. However, different *B. subtilis* strains had different effects on disease prevention (Zhao et al. 2014; Chandrasekaran et al. 2017).

In our previous study, 48 strains of *B. subtilis* were isolated from cotton endophytes to control cotton Verticillium wilt, and a strain EBS03 with good biocontrol potential was screened out (Bai et al. 2021). However, its mechanism of Verticillium wilt controlling remains unclear. Therefore, the inhibitory effect of EBS03 on *V. dahliae* and its immune induction function in cotton were studied *in vivo* and *in vitro*. These findings could provide new antagonistic resources for the biological controlling against plant diseases.

Materials and methods

Microbial strains and cotton varieties

B. subtilis EBS03 was screened from the cotton biocontrol bacteria population resource database established by our research group (Yuan et al. 2017; Bai et al. 2021; Wei et al. 2021), stored in frozen glycerol (− 80 ℃) and cultured in Luria-Bertani (LB) medium at 37 ℃. The strong virulence *V. dahliae* strain Vd080 was used to infect cotton, which was separated from the diseased soil in Xinji City, Hebei Province, China (37°56′ N, 115°15′ E). Upland cotton (*Gossypium hirsutum*) cultivar used in the experiment was Lumianyan 21, which was tolerant to Verticillium wilt (Li et al. 2009).

Detection of the antagonistic effect of EBS03 on *V. dahliae*

Preparation of fermentation broth

B. subtilis EBS03 was activated on LB solid medium and a single colony was picked in LB liquid medium at 37 °C, 150 r·min^{-1} shaking culture, when the fermentation broth of *B. subtilis* OD_{600} value (absorption value at the 600 nm wavelength) was 0.9 under spectrophotometer for use. A diameter of 5 mm block with *V. dahliae* strain Vd080 was transferred to the Czapek liquid medium at 25 ℃, 150 r·min^{-1} shaking culture for further use.

Nonvolatile metabolite inhibitory assay

First, a 5 mm (diameter, the same as below) block of Vd080 was taken in the center of 100 (Φ) × 15 (height) mm potato dextrose agar (PDA) medium; then, two Oxford cups were symmetrically placed at 20 mm from the center of the medium, and 50 μL of *B. subtilis* EBS03 was added to each cup. PDA medium with a 5 mm Vd080 block but without EBS03 was used as the control. Both treatments were incubated at 25 ℃, and each treatment was repeated 5 times. Ten days after incubation, the colony diameter of Vd080 was determined by the crisscross method; meanwhile, the colony morphology of Vd080 was recorded and the inhibition rate (*I*) of EBS03 against *V. dahliae* was calculated according to the following formula: $I = [(D_1 - 5) - (D_2 - 5)] / (D_1 - 5) \times 100\%$ (Bai et al. 2021), where D_1 = average diameter of the fungal mat of control treatments (mm) and D_2 = average diameter of the fungal mat of EBS03 treatments (mm).

Volatile metabolite inhibitory assay

The effect of EBS03 volatile metabolite on *V. dahliae* was detected by the double buckle culture method. First, 5 μL of EBS03 was inoculated in PDA medium, and another PDA plate containing 5 mm block of Vd080 was placed inversely over the PDA medium with EBS03, but without EBS03 as the control. Then, both treatments were incubated at 25 ℃ and repeated 5 times. The colony morphology of Vd080 was recorded, and the inhibition rate of EBS03 against *V. dahliae* was calculated as described above.

Inhibition of EBS03 on sporulation of V. dahliae

First, 10 mL of EBS03 fermentation broth was added to a 50 mL volume triangular flask, and then 10 mL spore suspension (1×10^7 mL^{-1}) of Vd080 was added. The

same volume LB liquid medium and the same concentration and volume spore suspension of Vd080 were added to another flask as the control. These flasks were shaken at 150 r·min^{-1} under 25 ℃. The sporulation of Vd080 was estimated at 48 h, 72 h, and 96 h with a hemocytometer, respectively. Each treatment was repeated 5 times.

Inhibition of EBS03 on germination of microsclerotia of V. dahliae

The EBS03 fermentation broth was centrifuged at 5 000 r·min^{-1} for 10 min at 4℃, and filtered with a 0.22 μm pore size filter to sterilize. A 100 μL of original filtrate, 1/2 dilution solution, and 1/4 dilution solution were evenly mixed with 100 μL of Vd080 spore suspension (1×10^7 mL^{-1}), respectively. The same volume mixture of LB medium and Vd080 spore suspension was used as the control. The germination of microsclerotia was detected under the microscope after 20 h in dark culture at 18℃. The germination rate was calculated through repeated 5 times in each treatment, and 100 microsclerotia were observed each time. The microsclerotia that the length of germ tube exceeded half of its length was considered as germinated microsclerotia (Zhou 2017).

Controlling effect of EBS03 against cotton verticillium wilt

Controlling effect of ESB03 on cotton verticillium wilt in the greenhouse

Seed soaking method: vermiculite, sand, and nutritious soil were mixed evenly according to the mass ratio at 3:2:1 and then put into a paper bowl (diameter 6 cm, height 10 cm) with 100 g total weight and 40% volumetric water content. Cotton seedling cultivation and pathogen inoculation method are same as the previous study (Zhu et al. 2010). The seeds of Lumianyan 21 were sterilized with 3% (mass fraction) sodium hypochlorite, and then soaked in EBS03 fermentation broth, with the concentration of $OD_{600} = 0.9$ (the same amount as the following method) for 12 h, then planted in a nutrition bowl with a density of 8 seeds per bowl. Six bowls were used as a group, 3 groups for each treatment, and the seeds soaked in LB medium were used as the control. When the first true leaf of cotton appeared, each bowl was inoculated with 10 mL of Vd080 spore suspension (1×10^7 mL^{-1}).

Root irrigation method: the disinfection and sowing methods of Lumianyan 21 were the same as above. When the first true leaf emerged, each bowl was inoculated with 10 mL of EBS03 fermentation broth. After 3 days of treatment, 10 mL of Vd080 spore suspension (1×10^7 mL^{-1}) was irrigated in each previously processed bowl, and the same amount of LB medium was irrigated as the control.

At 15 days after inoculation of Vd080, the symptoms of Verticillium wilt were observed and measured, and the disease index (DI) and control efficacy of EBS03 were calculated followed previous studies (Zhao et al. 2017; Zhang et al. 2021). At 60 days after cotton sowing, 15 cotton plants were randomly selected to measure the biomass indexes such as plant height, root length, fresh weight of plant (Bai et al. 2021).

Controlling effect of ESB03 on cotton verticillium wilt in the field

Lumianyan 21 was planted in the disease nursery (Anyang, Henan, China, 36°06′ N and 114°45′ E) where Verticillium wilt occurred seriously. The planting plot was designed according to the experiment. Each zone was 3.3 m long and 2.8 m wide, and the plant distance was 20 cm. Each treatment was repeated 3 times.

Seed soaking method: Lumianyan 21 seeds were soaked in EBS03 medium for 12 h, then planted at the density of 150 seeds in each plot. The seeds soaked with LB medium were used as the control (Zhou 2017). The emergence rate of cotton was calculated at 10 days after sowing, and 15 cotton seedlings were randomly selected at 25 days after sowing to measure root length, plant height, fresh weight of plant. Root irrigation method: after the emergence of cotton seedlings, each plot was irrigated twice with 0.8 L of EBS03 fermentation broth and the irrigation time interval for 20 days, using the same amount of LB medium irrigation for twice as the control. Spraying method: each plot was sprayed twice with 0.4 L of EBS03 fermentation broth and spraying time interval for 20 days, using the same amount of LB medium spraying for twice as the control. The symptoms of Verticillium wilt were observed and measured, and the disease index and control efficacy of EBS03 were calculated followed the previous study (Zhao et al. 2017). Furthermore, the plant height, fruit branch number, boll number per plant, 30 boll seed weight (boll weight), and 30 boll lint weight (lint weight) were determinated during the cotton harvest.

Mechanism of EBS03 in controlling cotton Verticillium wilt

Detection of resistance to V. dahliae in cotton leaf induced by EBS03

The seed soaking and sowing methods of Lumianyan 21 were the same as above. When the third true leaf appeared, the cotton roots were irrigated with 10 mL of EBS03 fermentation broth. After 48 h, the true leaves were disinfected on the surface and placed in a water agar medium. Every leaf was inoculated with 5 mm block of Vd080 and cultured at 25℃. After 7 days, the colony

phenotype of Vd080 was observed. The same amount of LB medium was irrigated as the control. Each treatment was repeated 5 times (Zhou 2017).

Determination of reactive oxygen species burst

When the third true leaf appeared, the cotton roots were irrigated with 10 mL of EBS03 fermentation broth, using the roots irrigation with the same amount of LB medium as the control. After 48 h, the true leaves with similar growth were washed with sterile water for three times and placed in the 50 mL centrifuge tube, then soaked in 20 mL of 3, 3′-diaminobenzidine (DAB) dye solution (1 g·L^{-1}, pH 7.5) and dyed for 8 h in dark at room temperature. Next, the dye solution was removed and the appropriate amount of 95% (volume fraction) ethanol was added to boiling water bath for 2 min to remove chlorophyll. Furthermore, 20 mL of anhydrous ethanol was added to the boiling water bath to continue decolorization, until the green coloration was completely removed. The leaves were then soaked in 70% (volume fraction) glycerol to remove the intercellular bubbles, and the leaves were placed on glass slides for microscopic observation (Zhou 2017).

Determination of callose accumulation

The true leaves of cotton were irrigated with EBS03 as above. The leaves were immersed in mixed solution of the 3:1 (volume ratio) ethanol and acetic acid for 3 h to remove the chlorophyll and then soaked in 70% (volume fraction) ethanol for 3 h and soaked overnight in sterile water. The next day, the leaves were gently rinsed and treated with 10% (mass fraction) NaOH for 2 h to make them transparent. Next, the leaves were washed with distilled water for 3 times, and then cultured in 0.01% (mass fraction) aniline blue for 3 h. Finally, the amount of callose was observed under fluorescence microscope (Yang et al. 2017).

Determination of defense-related genes expression

When the first true leaf of cotton appeared, the roots were irrigated with EBS03 fermentation broth, and inoculated with 10 mL of Vd080 spore suspension (1×10^7 mL^{-1}) in each bowl after 3 days. At 24, 48, and 72 h post inoculation (hpi), cotton leaves were collected to extract RNA and reverse transcribed into cDNA for qPCR detection. The qPCR reaction system and reaction conditions were the same as preliminary study (Pu et al. 2022). The highly conserved *ubiquitin* gene in cotton was taken as the internal reference, while the defense-related genes such as *POD*, *PPO*, *PAL*, and *PR10* were used as target genes. The specific primers of these genes in cotton were shown in Table 1.

Determination of V. dahliae colonization

When the first true leaf of cotton appeared, the roots were irrigated with EBS03 fermentation broth, and inoculated with 10 mL of Vd080 spore suspension (1×10^7 mL^{-1}) in each bowl after 3 days. Then, the hypocotyls of cotton were collected at 5 days after inoculation to detect the colonization of *V. dahliae*. The highly conserved gene *ubiquitin* in cotton was used as the internal reference, and the *β-tubulin* gene of *V. dahliae* was used as the target gene. The expression level of *β-tubulin* gene was detected by qPCR, so as to calculate the colonization of Vd080. The qPCR reaction system and reaction conditions were the same as preliminary studies (Sun et al. 2014; Zhang et al. 2015). The specific primers of two genes were shown in Table 1.

Statistical analyses

Data analyses were performed using Microsoft Excel 2019, and statistically analyzed using SPSS 26 by one way analysis of variance (ANOVA). The means ± standard deviation were compared by Duncan test and the difference significances were analyzed at $P < 0.05$ level.

Results

Inhibitory effect of nonvolatile metabolite of EBS03 on *V. dahliae*

The results of the confrontation test showed that the colony growth of Vd080 in the control was normal, while in the treatment with EBS03, the colony growth was inhibited, with the inhibitory effect of 72.39%, and interestingly, microsclerotia appeared (Fig. 1). The results showed that the nonvolatile metabolite of EBS03 could inhibit the growth of *V. dahliae*.

Table 1 Specific primers sequence of defense-related genes, the internal reference and *β-tubulin*

Gene name	Primer sequence(5′ − 3′)	
POD	F: CCGCATAACCATCACAAG	R: ACTCTCATCACCTTCAACA
PPO	F: ATATCCTTGTTCTGTCTGCTA	R: CTCCTTCTACCGTCTCTTC
PAL	F: TGGTGGCTGAGTTTAGGAAA	R: TGAGTGAGGCAATGTGTGA
PR10	F: ATGATTGAAGGTCGGCCTTTAGGG	R: CAGCTGCCACAAACTGGTTCTCAT
β-tubulin	F: AACAACAGTCCGATGGATAATTC	R: GTACCGGGCTCGAGATCG
ubiquitin	F: GAGTCTTCGGACACCATTG	R: CTTGACCTTCTTCTTCTTGTGC

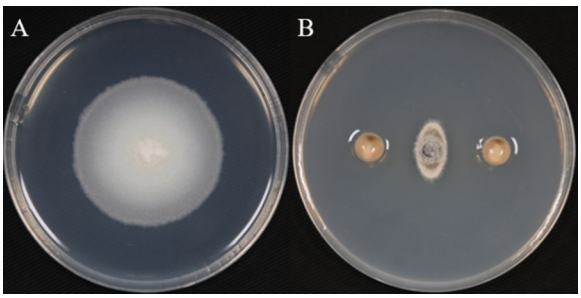

Fig. 1 Inhibitory effect of nonvolatile metabolite of EBS03 on *V. dahliae*. **A** control; **B** treated with EBS03

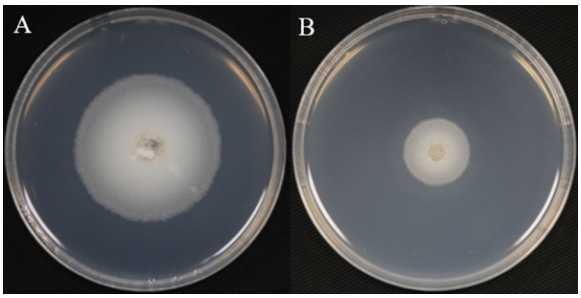

Fig. 2 Inhibitory effect of volatile metabolite of EBS03 on *V. dahliae*. **A** control; **B** treated with EBS03

Inhibitory effect of volatile metabolite of EBS03 on *V. dahliae*

The results of the double buckle culture test showed that the colony diameter of Vd080 treated with EBS03 was 17.60 mm at 10 days after incubation, and the inhibitory effect was 62.79%, indicating that the volatile metabolites of EBS03 also inhibit the growth of *V. dahliae* (Fig. 2).

Inhibitory effect of EBS03 on sporulation of *V. dahliae*

EBS03 treatment reduced the spore yield of *V. dahliae*, and the inhibitory effects of EBS03 against Vd080 were 54.01%, 47.37%, and 40.10% at 48, 72, and 96 h, respectively, and the inhibition effect gradually decreased with the culture time (Fig. 3), indicating that EBS03 could inhibit the production of sporulation of *V. dahliae*.

Inhibitory effect of EBS03 on microsclerotia germination of *V. dahliae*

After co-culture of microsclerotia of Vd080 and EBS03 in the dark for 20 h, the germination of microsclerotia was observed and calculated. The results showed that the germination rates of Vd080 with 3 different concentrations of EBS03 were 8.80%, 11.80%, and 16.41%, respectively, compared with 41.21% of the control treatment, and the inhibitory effects were 78.64%, 71.36%, and 60.19%, respectively (Fig. 4).

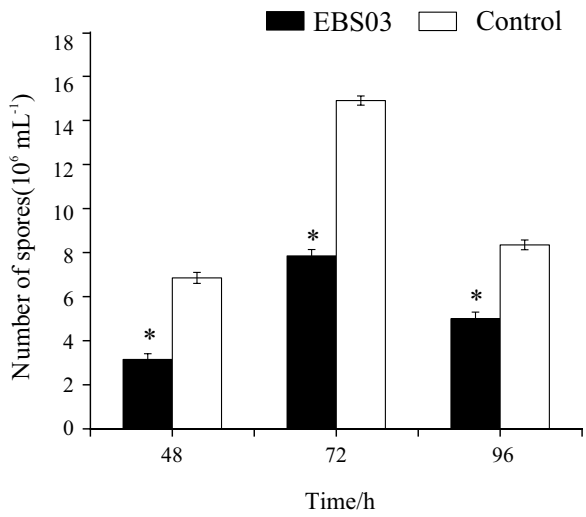

Fig. 3 Inhibitory effect of EBS03 on sporulation of *V. dahliae*

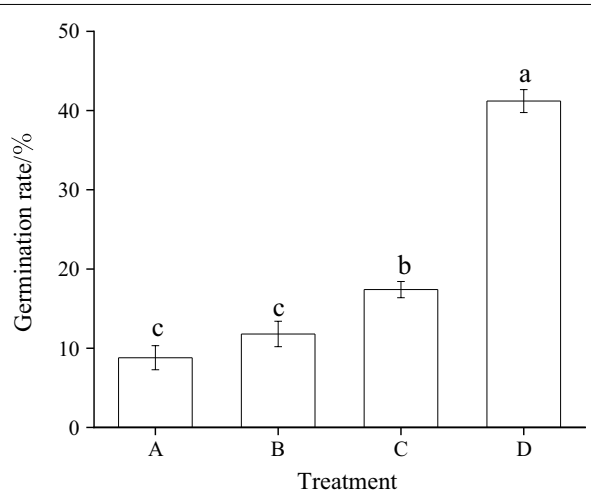

Fig. 4 Effect of EBS03 on microsclerotia germination of *V. dahliae*. **A** original filtrate of EBS03; **B** 1/2 dilution; **C**: 1/4 dilution; **D** LB medium

EBS03 had controlling effect against cotton Verticillium wilt in the greenhouse

In the greenhouse test, both seed soaking and root irrigation with EBS03 significantly reduced the diseased plant rate and disease index of cotton compared with the control, and the controlling effects of seed soaking and root irrigation with EBS03 reached 76.33% and 87.11%, respectively (Table 2). The preliminary results showed that EBS03 had good controlling effect against cotton Verticillium wilt in the greenhouse, which still needed to be varified by field tests.

Table 2 Controlling effects of different treatments of EBS03 against cotton Verticillium wilt in the greenhouse

Treatment	Diseased plant rate/%	Disease index	Control efficacy/%
Seed soaking	4.17*	8.17 ± 3.46*	76.33 ± 10.01
Seed soaking control	31.95	34.51 ± 4.71	/
Root irrigation	4.17*	3.84 ± 2.30*	87.11 ± 7.72
Root irrigation control	16.66	29.78 ± 2.31	/

Data are mean ± SD; * indicates that there is a significant difference between the treatment and the control ($P < 0.05$)

EBS03 promoted the growth of cotton in the greenhouse

As shown in Table 3, compared with the control, the root length, plant height, aboveground fresh weight of plant, and total fresh weight of plant were significantly increased by 35.00% and 43.39%, 78.58% and 78.08%, 69.14% and 113.33%, 69.32% and 107.23%, respectively, under the treatments of seed soaking and root irrigation with EBS03. However, the emergence rate of cotton with EBS03 treatment had no significant difference compared with the control. These results indicated that EBS03 increased the growth and development of cotton seedlings.

EBS03 had controlling effect against cotton Verticillium wilt in the field

In the field test, at 60 days after sowing, seed soaking, root irrigation and spraying with EBS03 all significantly reduced the disease index of cotton Verticillium wilt compared with the control, and the controlling effect of seed soaking, root irrigation and spraying with EBS03 reached 24.98%, 31.18% and 42.54%, respectively. With the extension of time, the controlling effects of ESB03 against cotton Verticillium wilt declined. However, there were still significant differences compared with the control. Among the three treatments, spraying with EBS03 had the best controlling effect, reaching 35.65% (Table 4).

EBS03 promoted the growth of cotton in the field

Similar to the greenhouse test, in the field, the emergence rate of cotton seedlings treated with EBS03 was 67.33%, which was significantly higher than that of the control (61.00%), and the root length of cotton seedlings treated with EBS03 significantly increased by 11.14%. Besides, compared with the control, there was no significant difference in other cotton biomass indexes (Table 5).

Table 3 Controlling effects of different treatments of EBS03 on cotton biomass indexes

Treatment	Emergence rate/%	Root length/cm	Plant height/cm	Aboveground fresh weight of plant/g	Total fresh weight of plant/g
Seed soaking	71.53	7.56 ± 0.23*	23.93 ± 0.28*	1.37 ± 0.01*	1.49 ± 0.02*
Seed soaking control	73.61	5.60 ± 0.27	13.40 ± 0.24	0.81 ± 0.01	0.88 ± 0.01
Root irrigation	/	8.13 ± 0.50*	22.67 ± 0.44*	1.60 ± 0.12*	1.72 ± 0.04*
Root irrigation control	/	5.67 ± 0.25	12.73 ± 0.37	0.75 ± 0.01	0.83 ± 0.01

Data are mean ± SD; * indicates that there is a significant difference between the treatment and the control ($P < 0.05$)

Table 4 Controlling effects of different treatments of EBS03 against cotton Verticillium wilt in the field

Treatment	60 days after sowing		80 days after sowing	
	Disease index	Control efficacy/%	Disease index	Control efficacy/%
Seed soaking	16.22 ± 0.23*	24.98 ± 1.07	22.36 ± 0.69*	17.82 ± 2.53
Seed soaking control	21.62 ± 0.91	/	27.21 ± 4.18	/
Root irrigation	12.76 ± 0.13*	31.18 ± 0.70	20.63 ± 0.27*	21.08 ± 1.04
Root irrigation control	18.54 ± 2.45	/	26.14 ± 0.90	/
Spraying	12.51 ± 0.18*	42.54 ± 0.83	16.82 ± 0.40*	35.65 ± 1.63
Spraying control	21.77 ± 0.15	/	26.14 ± 0.90	/

Data are mean ± SD; * indicates that there is a significant difference between the treatment and the control ($P < 0.05$)

Table 5 Effect of EBS03 on seedling biomass indexes of cotton

Treatment	Emergence rate/%	Root length/cm	Plant height/cm	Aboveground fresh weight of plant/g	Total fresh weight of plant/g
Seed soaking	67.33 ± 7.69 *	7.58 ± 0.36 *	19.39 ± 0.47	34.28 ± 0.28	38.09 ± 0.76
Seed soaking control	61.00 ± 3.79	6.82 ± 0.22	20.13 ± 0.47	34.37 ± 0.60	37.80 ± 0.71

Data are mean ± SD; * indicates that there is a significant difference between the treatment and the control ($P < 0.05$)

Table 6 Effects of different treatments of EBS03 on cotton yield

Treatment	Plan height/cm	Fruit branch number	Boll number per plant	30 boll seed cotton weight/g	30 boll lint weight/g
Seed soaking	80.13 ± 0.13*	15.13 ± 0.24	11.47 ± 1.92*	162.20 ± 0.56*	62.80 ± 1.63*
Seed soaking control	71.20 ± 4.02	14.07 ± 0.29	9.60 ± 0.20	151.00 ± 2.05	60.50 ± 1.47
Root irrigation	88.53 ± 1.74*	16.40 ± 0.50*	15.80 ± 1.03*	173.77 ± 1.93*	66.87 ± 1.33*
Root irrigation control	77.87 ± 0.74	15.33 ± 0.35	12.07 ± 1.27	169.33 ± 2.05	64.93 ± 0.90
Spraying	81.33 ± 0.64*	16.20 ± 0.12*	14.07 ± 0.24*	178.83 ± 3.27*	67.00 ± 2.16*
Spraying control	70.47 ± 0.07	13.87 ± 0.18	12.13 ± 0.07	163.77 ± 2.88	64.33 ± 1.70

Data are mean ± SD; * indicates that there is a significant difference between the treatment and the control ($P < 0.05$).

Table 7 Effects of different treatments of EBS03 on cotton fiber quality

Treatment	Upper half mean length/mm	Uniformity index/%	Specific strength to break / (cN·tex^{-1})	Micronaire	Breaking elongation/%
Root irrigation	30.00	86.30	30.50	5.00	6.80
Root irrigation control	29.00	84.20	27.90	5.30	6.70
Spraying	29.30	86.00	31.60	4.80	6.80
Spraying control	27.00	85.00	29.60	5.10	6.70

EBS03 promoted the yield and quality of cotton in the field

In the field experiment, compared with the control, boll number per plant and 30 boll seed cotton weight were significantly increased by 19.48% and 7.42%, 30.90% and 2.62%, 15.99% and 9.20%, respectively, under the treatments of seed soaking, root irrigation, and spraying with EBS03 (Table 6). Both biological indicators were important factors in cotton yield. Interestingly, fiber quality of cotton treated with root irrigation and spraying with EBS03 also increased (Table 7), but due to the small sample size, no significant difference was analyzed. Collectively, these results suggest that ESB03 promoted cotton yield and fiber quality, and had a good application prospect.

Resistance of cotton leaf to the infection of *V. dahliae* induced by EBS03

After EBS03 root irrigation treatment, the true leaves of cotton improved the resistance to infection of *V. dahliae* *in vitro*. After 7 days, it was observed that there was little mycelium colonization of Vd080 on the leaf surface and the necrotic area was small. Compared with the control, there were a large number of mycelium colonization on the leaf surface, and the necrotic area was larger (Fig. 5).

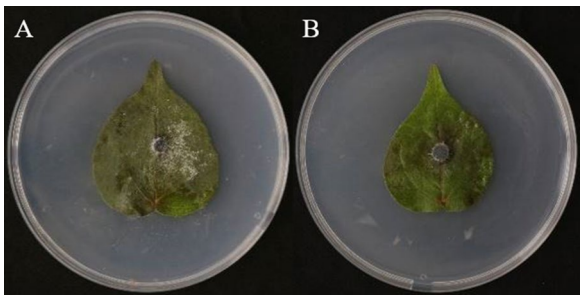

Fig. 5 EBS03 enhanced cotton leaves against *V. dahliae*. **A** control; **B** treated with EBS03

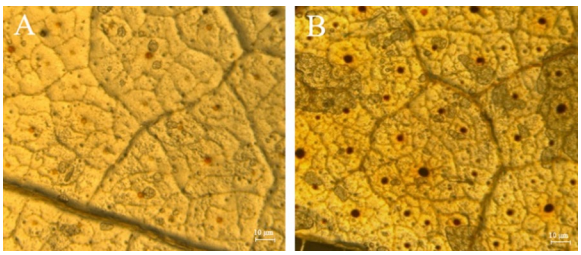

Fig. 6 EBS03 triggered the outbreak of reactive oxygen species in cotton leaf. **A** control; **B** treated with EBS03

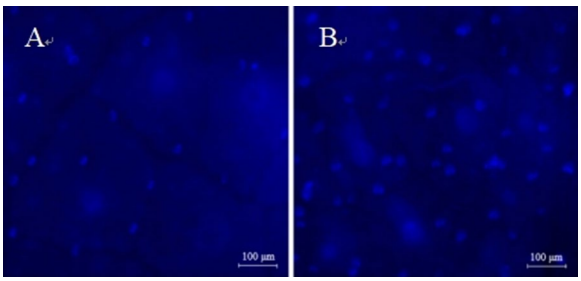

Fig. 7 EBS03 induced callose accumulation in cotton leaves. **A** control; **B** treated with EBS03

The outbreak of reactive oxygen species in cotton leaf induced by EBS03

Compared with the control, more brown precipitation was observed in the leaf treated with EBS03 root irrigation, indicating that EBS03 triggered the outbreak of reactive oxygen species (ROS) in cotton leaf (Fig. 6).

Callose accumulation in cotton leaf induced by EBS03

The determination of callose accumulation in cotton leaf was detected after treated with EBS03 for 2 days. Compared with the control, the accumulation of callose in cotton leaves treated with EBS03 was higher, about twice to the control, indicating that EBS03 induced the accumulation of callose in cotton leaf (Fig. 7).

The expression level of defense-related genes in cotton induced by EBS03

The expression level of defense-related genes *POD*, *PPO*, *PAL*, and *PR10* in cotton leaf were detected by RT-qPCR. The results showed that EBS03 significantly induced the upregulation expression level of defense-related genes. Among them, the expression of *POD* and *PPO* in EBS03 treatment reached the peak level at 72 hpi, which were 6.16 and 2.12 times than these of the control, respectively. While the expression level of *PAL* at 48 hpi was the highest, with 1.74 times than that of the control. At 24 hpi, the expression level of *PR10* in EBS03 treatment was 1.62 times of the control (Fig. 8).

EBS03 reduced the colonization level of *V. dahliae* in cotton

The colonization level of *V. dahliae* in hypocotyl of cotton was an important reason for cotton Verticillium wilt. At 5 days after inoculation, compared with the control, the colonization level of *V. dahliae* in hypocotyl of cotton treated with EBS03 was significantly reduced, with one-third of the control (Fig. 9). These results showed that EBS03 reduced the colonization level of *V. dahliae* in cotton.

Discussion

Up to now, *B. subtilis* had played an increasingly important role in the controlling of plant diseases. However, due to different strains and sources, their control effects varied greatly (Li et al. 2005; Zhao et al. 2014). As an endophytic bacteria, *B. subtilis* EBS03 isolated from cotton plant, with no negative effect on cotton, had a promising application in the controlling of cotton Verticillium wilt (Bai et al. 2021). Many studies on the antagonism of biocontrol microbial metabolites against plant disease pathogens showed that volatile and nonvolatile metabolite of biocontrol microbe directly inhibited the growth of pathogenic fungal mycelium (Kai et al. 2007; Sharifi et al. 2016). The volatile and nonvolatile metabolite of EBS03 also inhibited the mycelium growth, sporulation, and microsclerotia germination of *V. dahliae*. However, the specific components of metabolite of EBS03 still needed to be further studied. The volatile metabolites could only have an antagonistic effect on pathogens at a certain concentration (Song and Ryu 2013), which may be one of reasons why the controlling effect of ESB03 on cotton Verticillium wilt in the greenhouse (relatively confined space) was better than that in the field.

In the controlling of soil-borne diseases, biological microbe have become the most environmentally friendly method, which was also an important choice to supplement the existing control measures (Sehrawat et al. 2022). Previous studies have shown that *B. subtilis* strains NCD-2, HMB19198, and BS-2 were widely used in the controlling of cotton Verticillium wilt (Li et al. 2005; Qu et al. 2022), but there were few studies on the systematic evaluation of the effect of *B. subtilis* on cotton growth and yield both in the greenhouse and the field. In this study, the treatment of seed soaking and root irrigation of ESB03 significantly promoted the root length, plant height, aboveground fresh weight of plant, and total fresh weight of plant in the greenhouse. Furthermore, as important components of cotton yield, the boll number per plant and boll weight in EBS03 fermentation broth

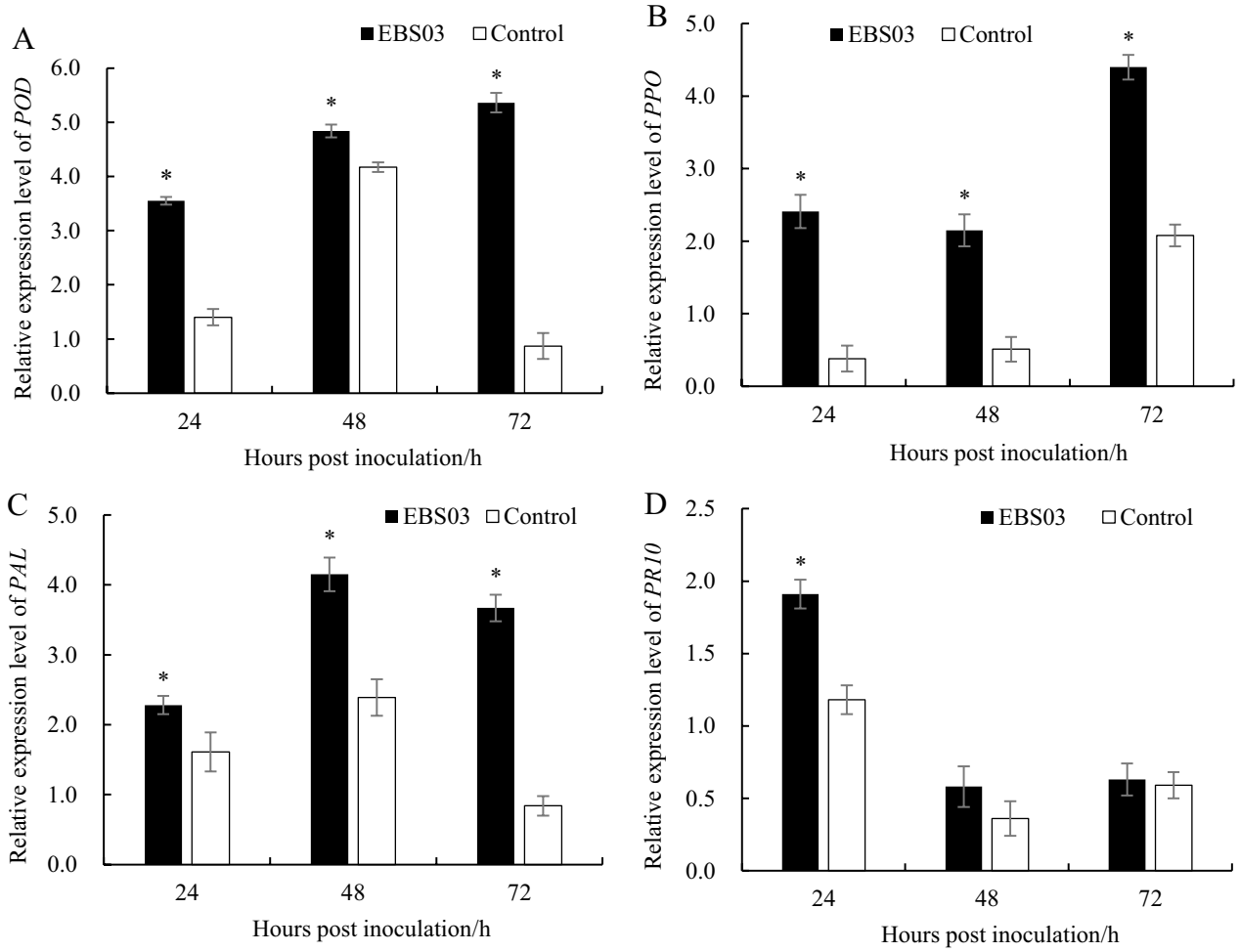

Fig. 8 EBS03 induced the expression level of defense-related genes in cotton. **A**–**D** expression level of *POD*, *PPO*, *PAL*, and *PR10* in cotton with EBS03 treatment or not. * indicates a significant difference at the 0.05 probability level ($P < 0.05$)

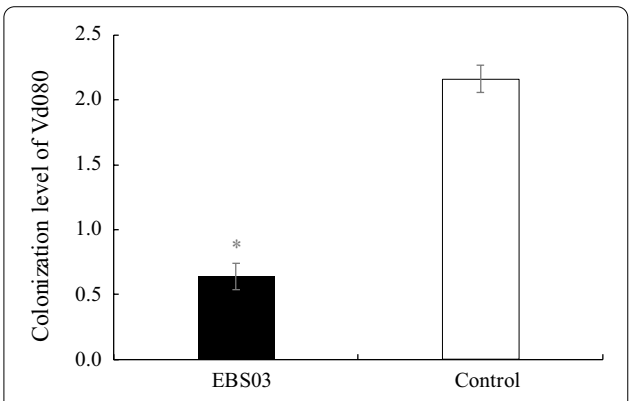

Fig. 9 Colonization level of *V. dahliae* in hypocotyl of cotton. * indicates a significant difference at the 0.05 probability level

seed soaking, root irrigation, and spraying treatments also significantly increased in the field. In the greenhouse, the controlling effect of ESB03 on cotton Verticillium wilt was more than 70%, however, the controlling effect of ESB03 in the field was declined with approximately 20%–40%. The reason may be that the biocontrol microbe was alive, and field environment and some abiotic factors affected the controlling effect of microbe (Li et al. 2005).

Bacillus subtilis controls plant disease mainly through competition and antibacterial metabolites, and induction of systemic disease resistance of plant. In most cases, multiple defense ways existed simultaneously (Henry et al. 2011; Zhao et al. 2022). In this study, on one hand, EBS03 induced the resistance of cotton leaf against the infection of *V. dahliae* and significantly decreased the colonization level of *V. dahliae* in cotton hypocotyl; on the other hand, ESB03 significantly increased the reactive oxygen species (ROS) and the accumulation of callose in cotton leaf. Furthermore, defense-related genes played an important role in plant resistance to pathogen infection (Yang et al. 2017). Phenylalanine aminolase (PAL) was a key enzyme in the synthesis of lignin and phenols, which

was important for the formation of plant disease resistance system (Millet et al. 2010; Lv et al. 2017). Besides, resistance-related protein gene *PR10* was a key disease-resistance gene of plants under various biotic and abiotic stresses (Jannoey et al. 2017). In this study, EBS03 induced the significantly upregulation expression level of defense-related genes *POD*, *PPO*, *PAL*, and *PR10.* Collectively, the controlling of cotton Verticillium wilt with ESB03 involved multiple regulatory ways, however, its control mechanism against cotton Verticillium wilt need to be further studied.

Conclusion

In this study, *B. subtilis* EBS03 effectively inhibited mycelium growth, sporulation, and microsclerotia germination of *V. dahliae*. In addition, ESB03 had a remarkable controlling effect on cotton Verticillium wilt, and could promote cotton growth and increase cotton yield both in the greenhouse and field. Furthermore, EBS03 induced cotton leaf resistance against *V. dahliae*, and increased the outbreak of reactive oxygen species and accumulation of callose, and significantly upregulated expression level of defense-related genes. Collectively, *B. subtilis* EBS03 has a good application prospect in the control of cotton Verticillium wilt.

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Authors' contributions
Conceptualization: Zhang YL, Peng J and Zhu HQ; Test operation: Bai HY; Writing-original draft: Bai HY, Feng ZL; Writing-review: Zhao LH, Feng HJ, Wei F, Zhou JL and Gu AX. All authors read and approved the final manuscript.

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Data Availability
All data generated or analyzed during this study are included in this published article and its additional files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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