Efficacy evaluation of Bacillus subtilis EBS03 on control of cotton Verticillium wilt

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Research

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Abstract

Background

In our previous study, we screened 48 strains of *Bacillus subtilis* from cotton endophytes for cotton Verticillium wilt control research and screened out a strain EBS03 with good biocontrol potential However, its mechanism for preventing Verticillium wilt remains unclear. The purpose of this study was to clarify its control mechanism to provide technical support for the control of cotton Verticillium wilt.

Results

The results of confrontation and buckle culture showed that the inhibition rates of *Bacillus subtilis* EBS03 on mycelium growth of *Verticillium dahliae* were 70.03% and 59.00%, respectively, and the inhibition rates of sporulation and microsclerotia germination were 47.16% and 70.06%, respectively. In the greenhouse test, the control effect of EBS03 root irrigation on cotton Verticillium wilt was the highest at 87.11%, significantly promoting the growth of cotton seedlings. In the field experiment, the control effect of EBS03 fermentation broth spray on cotton Verticillium wilt was 42.54% (60 days), among them, the quality of seed cotton and lint cotton in the soaking, root irrigation, and spraying treatments increased by 7.42% and 3.80%, 2.62% and 2.98%, 9.20% and 4.14%, respectively, compared with the control. In the induced resistance test, EBS03 improved the resistance of cotton leaves to *V. dahliae* infection and induced the outbreak of reactive oxygen species and callose accumulation. In addition, the results of qPCR detection showed that EBS03 induced up-regulation of defense-related genes such as *PAL*, *POD*, *PPO* and *PR*10, enhanced plant resistance to this fungal pathogen, and inhibited the reproduction of *V. dahliae* in cotton.

Conclusion

Bacillus subtilis EBS03 has a good biological defense capability by inhibiting the growth of *V. dahliae*, activating the disease resistance of the cotton, enhancing the disease resistance, and increasing cotton yield.

Introduction

Cotton is an important cultivated plant in China and around the world. Cotton Verticillium wilt, mainly caused by *Verticillium dahliae* Kleb., is a widespread disease that occurs in most cotton-producing areas, the disease can occur throughout the reproductive period (Chi et al. 2021; Fradin et al. 2010). Due to the stable dormant structure of microsclerotia, this fungus can survive in the soil for more than ten years, the pathogen population is rich in genetic diversity and its pathogenicity is prone to variability (Zhang et al. 2022). Therefore, cotton Verticillium wilt has not been effectively controlled. At present, the prevention and control of cotton Verticillium wilt at home and abroad mainly adopt traditional methods such as crop

rotation, selection of disease-resistant varieties, and chemical control, but the disease prevention effect is not ideal (Zhang et al. 2021; Zhao et al. 2017).

With the rise of green agriculture and organic agriculture, comprehensive control measures based on biological control have been paid more and more attention, It is very important in the prevention and treatment of diseases (Mitra et al. 2022; Acharya et al. 2020; Ingram et al. 2020). Among them, it is a research hotspot to screen antagonistic microorganisms from soil and cotton plant endophytes to control the disease (Berg et al. 2001; Bubici et al. 2013). At present, microorganisms with biological control ability have been found in different kinds of bacteria, fungi, and actinomycetes (Ku et al. 2018), mainly by inducing plant systemic resistance, further competing for nutrients and planting space, or by producing plant hormones and providing nutrients to inhibit pathogen infection and promote plant growth (Sehrawat et al. 2022; Varo et al. 2016). As an important group of biocontrol microorganisms, biocontrol bacteria play an important role in disease control, *Bacillus* sp. There are many applications, such as *Bacillus subtilis*, *Bacillus cereus*, *Bacillus pumilus*, *Bacillus licheniformis* and so on. *Bacillus subtilis* is widely used to prevent and treat agricultural diseases because of its strong adaptability and good antibacterial activity in the soil (Zhao et al. 2014; Chandrasekaran et al. 2017).

In our previous study, we screened 48 strains of *Bacillus subtilis* from cotton endophytes for cotton Verticillium wilt control research and screened out a strain EBS03 with good biocontrol potential (Bai et al. 2021). However, its mechanism for preventing Verticillium wilt remains unclear. Therefore, in this study, We examined the effects of EBS03 on the hyphae, sporulation, and microsclerotia germination of *V. dahliae*; furthermore, the control effect of EBS03 on cotton Verticillium wilt and the promotion effect on cotton growth were tested in greenhouse test and field test; in addition, we also determined the induction of EBS03 on the resistance response of cotton, including the expression of resistance-related genes, reactive oxygen species burst, and callose accumulation. The objective of this study is to reveal the mechanism of EBS03 inducing disease resistance in cotton and to provide new antagonistic resources for the biological control of cotton Verticillium wilt.

Materials And Methods

Microbial material

B. subtilis EBS03 comes from the cotton biocontrol bacteria population resource database established by our research group (Yuan et al. 2017; Wei et al. 2021). The isolated bacterium was stored as a frozen glycerol stock (−80 ℃) and maintained Luria-Bertani (LB) at 37 ℃. The strongly virulent defoliating *V. dahliae* strain Vd080 was used to infect cotton, which was separated from the diseased soil in Xinji, Hebei province, China (37˚56’N, 115˚15’E). Upland cotton (*Gossypium hirsutum*) cultivars, Lumianyan 21 (tolerance), were used in the tests (Li et al. 2009).

Detection of the antagonistic effect of endophytic bacteria on *V. dahliae*

Preparation of fungal spore and bacterial cell suspensions

B. subtilis EBS03 was activated on LB solid medium and a single colony was picked and placed in LB liquid medium and maintained at 37 ℃, 150 r·min^{-1}, after 24 h of shaking culture, the cultured broth of *B. subtilis* was obtained for use. A diameter of 5 mm of the fungal colony of Vd080 was taken and transferred to the Czapek solution at 25 ℃, 150 r·min^{-1}, after 7 days of shaking culture, the Vd080 spore suspension was obtained for use.

Nonvolatile metabolite inhibitory assay

In vitro confrontation, bioassays were performed on PDA by placing a 5 mm plug of actively-growing mycelia in the center of a 100 × 15 mm petri dish. Then, two oxford cups were symmetrically placed 20 mm from the center of the medium, and 50 μL of *B. subtilis* was added to each cup. Controls were prepared by placing a fungal plug in the center of a PDA dish without bacterial inoculations. All petri dishes were incubated at 25 ℃, and each treatment was repeated 5 times, after 10 days of incubation, determination of colony diameter of *V. dahliae by* the crisscross method. Following incubation, growth was recorded and the percentage of inhibition was calculated according to the following formula: percent inhibition (*I*): $I = [(D_1 - 5) - (D_2 - 5)] / (D_1 - 5) \times 100$ (China National Institute of Standardization. 2020), where D_1 = average diameter of the fungal thallus of control treatments (mm) and D_2 = average diameter of the fungal thallus of *B. subtilis* EBS03 treatments (mm).

Volatile metabolite inhibitory bioassay

Inhibition of *V. dahliae* mycelial growth by volatiles of EBS03 was tested using the buckle culture method. Inoculated 5 μL of *B. subtilis* EBS03 in one fresh PDA petri dish, and another fresh PDA petri dish containing a mycelial plug (5 mm diameter) of *V. dahliae* Vd080 was placed inversely over the petri dish containing the culture of EBS03; this double-dish set was immediately sealed with parafilm. In the control treatment, a PDA petri dish inoculated with *V. dahliae* Vd080 was placed inversely over another petri dish containing LB but without EBS03 to make a double-dish set. Each treatment was repeated 5 times. The diameter of the *V. dahliae* Vd080 colony in each double-dish set was measured 10 days after incubation at 25 ℃, and the percentage of growth inhibition was calculated using the method described above.

Antifungal activity of *B. subtilis* EBS03 on sporulation of *V. dahliae.*

Culture filtrate of EBS03 (10 mL) was added to a 50 mL sterile erlenmeyer flask, and then 10 mL spore suspension (1 × 10^7 spores·mL^{-1}) of Vd080 cultured in liquid Czapek medium was added. The same volumes of liquid LB medium and spore suspension of Vd080 were added to another flask as the control. The flasks were shaken at 150 r·min^{-1} for 48, 72 and 96 h at room temperature (25 ℃). Spore concentrations were estimated with a hemocytometer. Each treatment was repeated 5 times.

B. subtilis EBS03 inhibited the germination of *V. dahliae* Vd080 microsclerotia

The endophytic bacterial culture was centrifuged at 5 000 r·min^{-1} for 10 min at 4 ℃. Filter the supernatant through a 0.22 μm filter to sterilize. Took 100 μL of sterile filtrate stock solution, 1/2 dilution

solution and 1/4 dilution solution, and mixed them evenly with an equal amount of Vd080 spore suspension (1×10^7 spore·mL^{-1}). The same volumes of liquid LB medium and spore suspension of Vd080 were added to another 1.5 mL centrifuge tube as the control. These experiments were incubated for 18 h at 18 ℃ in the dark. Following the incubation period, Germination rates were observed under visible light using an inverted microscope. Observe the germination of 100 microsclerotia each time. Each treatment was repeated 5 times, microsclerotia was considered germinated when the length of the germ tube equaled at least the length of the microsclerotia.

Control effect of strain EBS03 on Verticillium wilt of cotton

A greenhouse experiment to determine the effect of control

Seed soaking method: vermiculite, sand and nutritious soil were mixed evenly according to the mass ratio at 3: 2: 1 and then loaded into a paper bowl (diameter 6 cm, height 10 cm). Cotton seedling cultivation and pathogen inoculation methods refer to our previous method (Zhu et al. 2010). At the same time, the cotton seeds of Lumianyan 21 were soaked in an EBS03 culture medium for 12 h, and 8 cotton seeds were sown in each paper bowl, one treatment per 6 paper bowls, each treatment was repeated 3 times, and the aseptic LB soaking seeds were used as the control. The seedlings were carried out 7 days after sowing, and 5 cotton seedlings were retained in each bowl. When a true leaf first appeared, each paper bowl was inoculated with 10 mL Vd080 spore suspension (1×10^7 spore·mL^{-1}). The cotton growth situation was tracked and the cotton Verticillium wilt disease was investigated in time.

Root irrigation method: soaking cotton seed Lumianyan 21 in warm soup for 12 h, sowing and fixing seedlings refer to the soaking method. After the first appearance of a true leaf of cotton, the roots were irrigated with 10 mL spore liquid of EBS03 culture medium. 3 days after treatment, each paper bowl was inoculated with 10 mL Vd080 spore suspension (1×10^7 spore·mL^{-1}), one treatment for every 6 paper bowls, each treatment was repeated 3 times, and the same amount of water was irrigated used as control.

15 days after inoculation of Vd080 spore suspension, the Verticillium wilt disease was investigated, and the disease index (*DI*) and disease prevention effect were calculated at the same time (Zhao et al. 2017; Zhang et al. 2021). At 60 days after sowing, 15 cotton plants were randomly selected to measure the biomass indexes such as plant height, root length, fresh matter quality and so on. The disease index of cotton Verticillium wilt was calculated as $DI = \Sigma (Ni \times I) / (N \times 4) \times 100$. In the formula, *DI* is the disease index, *Ni* is the number of diseased plants at all levels, *I* is the number of disease grades, and *N* is the total number of plants investigated. The formula for calculating the control effect (*E*) is $E\,(\%) = (DI_0 - DI_1) / DI_0 \times 100$, DI_0 is the control disease index and DI_1 is the treatment disease index.

A field experiment to determine the control effect

The disease-tolerant variety Lumianyan 21 was planted in the disease nursery 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 experiment was set to repeat 3 times.

Soaking seeds in EBS03 fermentation broth: soaking appropriate amount of sterilized seeds in EBS03 culture solution for 12 h, washing seeds with sterile water many times before sowing, counting the emergence number of Lumianyan 21 seeds, and calculating seedling emergence rate at 10 days after sowing, 15 cotton seedlings were randomly selected from each treatment at 25 days after emergence to measure the root length, plant height and fresh material quality. Root irrigation with EBS03 fermented liquid: after cotton emergence, EBS03 culture liquid was irrigated on the cotton root with 0.8 L per zone and sprayed on the cotton seedling. EBS03 fermentation liquid spray: the prepared EBS03 fermentation liquid was sprayed on cotton seedlings, and sprayed once every 20 days, a total of 2 times, 0.4 L per zone, with the same amount of water spray as the control. The classification standard, disease index, and control effect of the Verticillium wilt disease were the same as the greenhouse experiment to determine the effective control of EBS03. Furthermore, the plant height, fruit branch number, boll number per plant, 30 boll seed quality, and 30 boll lint quality were investigated during the cotton harvest.

Study on the mechanism of strain EBS03 controlling Verticillium wilt of cotton

Detection of resistance of cotton leaves induced by strain EBS03

Soaked the sterilized seeds in an activated EBS03 medium for 12 h, rinsed the seeds with sterile water 2 times, and sowed 8 seeds in a paper bowl. After the cotton seedlings grew to 3 true leaves, they were irrigated with an EBS03 culture medium for root treatment. After 2 days, the true leaves of cotton were taken for surface disinfection, then washed with sterile water 2 times, placed on the surface of a water agar plate, and each leaf was inoculated with 5 mm of the fungal colony of *V. dahliae*. The leaves were cultured at 25 ℃ for 7 days, and the damage to leaves was observed. Inoculated with LB liquid medium alone as a control, each treatment was repeated 5 times.

Detection of reactive oxygen species burst in cotton leaves induced by strain EBS03

When 2 true leaves of cotton were grown, the roots were irrigated with 10 mL of an EBS03 culture medium per bowl. 2 days after inoculation, the eruption and accumulation of reactive oxygen species in cotton leaves were detected by 3, 3’-diaminobenzidine (DAB) tissue staining method. The true cotton leaves with similar growth were washed with sterile water and placed in 50 mL centrifuge tubes. An appropriate amount of DAB staining solution (1 $g \cdot L^{-1}$, pH 7.5) was added to the centrifuge tube and stained at room temperature for 8 h. After removing the dye solution, added an appropriate amount of 95% ethanol in the boiling water bath for 2 min to remove chlorophyll, remove the liquid add the appropriate amount of anhydrous ethanol to continue decolorization until the green leaves were completely removed. Finally, the leaves were soaked in 70% (volume fraction) of glycerol, the intercellular bubbles were driven out, and the leaves were placed on glass slides and observed by fluorescence microscope Nikon80i.

Determination of callose deposition in cotton plants induced by strain EBS03

When the cotton seedlings grow 2 true leaves, inoculate the culture solution of strain EBS03, 10 mL per pot. After inoculation with EBS03 medium for 2 days, the cotton true leaves with similar growth were washed with sterile water and placed in a 50 mL centrifuge tube. The whole leaves were fixed in a fixed solution with a volume ratio of ethanol to acetic acid at 3: 1 for 3 h, and chlorophyll was removed. Soaked in 70% and 50% ethanol for 3 h, then soaked in sterile water overnight. After pouring water the next day, rinsed the leaves gently and treated the leaves in 10% (mass fraction) NaOH for 2 h to make the leaves transparent. The leaves were washed with distilled water 4 times, then cultured in 0.01% (mass fraction) aniline blue for 3 h, and finally, the amount of callose was observed by fluorescence microscope Nikon80i.

Determination of defense-related gene expression in cotton induced by strain EBS03 After the first true leaf of cotton appeared, the roots were irrigated with an EBS03 culture medium. 3 days after treatment, 10 mL Vd080 spore suspension (1×10^7 spore·mL^{-1}) was inoculated in each paper bowl, and the root was irrigated with the same amount of water as the control, each treatment was repeated 3 times. At 24 h, 48 h and 72 h after the infection of cotton by *V. dahliae*, the leaves of cotton were collected to detect the expression of defense-related genes. RNA was extracted from cotton leaves by RNAprepPurePlantKit (TIANGEN). The concentration of RNA was detected by NanoDrop 2000, and the concentration of RNA was adjusted to 100 ng·μL^{-1}. According to Zhang et al (2016) method, the expression of defense-related genes peroxidase (*POD*), polyphenol oxidase (*PPO*), phenylalanine aminolase (*PAL*), and disease-resistance-related gene (*PR*10) in cotton leaves were detected. The highly conserved gene *Ubiquitin* in cotton was used as the internal reference, and the relative expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Huang et al. 2021). The specific primers of defense genes were shown in Table 1.

Table 1 Specific primer sequences of related resistance genes

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

Detection of *V. dahliae* colonization in cotton tissue by qPCR

After the first true leaf of cotton appearances, the roots were irrigated with an EBS03 culture medium, and 10 mL Vd080 spore suspension (1×10^7 spore·mL^{-1}) was inoculated in each paper bowl after 3 days. The hypocotyls of cotton seedlings were collected 5 days after inoculation. The qPCR reaction system and

reaction conditions were the same as described above. The highly conserved gene *Ubiquitin* in cotton was used as the internal reference gene, and the *β-tubulin* gene of *V. dahliae* was used as the detection gene (Zhang et al. 2015). Determination of *V. dahliae* biomass in cotton hypocotyls (Sun et al. 2014).

Statistical Analyses

Data entry and analysis were performed using Microsoft Excel 2019. All the data were statistically analyzed using SPSS version 26 by one-way analysis of variance (ANOVA). The obtained means ± standard deviation were compared by Duncan's post hoc multiple range test and were considered significant at $p < 0.05$.

Results

Inhibitory effect of non-volatile metabolites of *B. subtilis* EBS03 on mycelial growth of *V. dahliae*

The results of the confrontation culture method showed that the growth of *V. dahliae* Vd080 was inhibited in the experimental group treated with strain EBS03, and the colony diameter was 11.10 mm when cultured for 10 days, compared to the colony diameter of Vd080 was 44.02 mm in the control, and the inhibition rate of strain EBS03 against *V. dahliae*Vd080 was 72.39%. The test results showed that the non-volatile metabolites of strain EBS03 had an inhibitory effect on the growth of *V. dahliae* Vd080 (Fig. 1).

Effects of *B. subtilis* EBS03 volatile metabolites on the growth of *V.dahliae* colonies

The results of the buckle culture method are shown in Fig. 2. On the plate without EBS03 inoculation, the Vd080 colony was white, with no microsclerotia and the growth was normal, while on the plate inoculated with EBS03, the colony growth was inhibited. The colony diameter was 17.60 mm after 10 days of culture, and the inhibition rate was 62.79%. The results showed that the volatile metabolites of strain EBS03 could inhibit the growth of *V. dahliae* Vd080.

Inhibitory effect of *B. subtilis* EBS03 aseptic filtrate on sporulation of *V. dahliae*

The results of the spore production test are shown in Fig. 3. EBS03 can inhibit the sporulation of *V. dahliae* after being treated for 48 h, 72 h and 96 h. The results of data analysis showed that the inhibition rates of the treatment group with EBS03 culture filtrate were 54.01%, 47.37% and 40.1%, respectively. The inhibitory effect of EBS03 culture medium on the sporulation of *V. dahliae* was significantly different, and the inhibitory effect on spore production was the best when co-cultured for 24 h. The above results showed that EBS03 aseptic filtrate had an inhibitory effect on the conidia production of *V. dahliae*.

Inhibitory effect of *B. subtilis* EBS03 culture medium on microsclerotia germination of *V. dahliae*

The microsclerotia of *V. dahliae* and EBS03 aseptic filtrate were co-cultured under dark conditions for 20 h, and the germination of microsclerotia was calculated. The results showed that the germination rate of

microsclerotia in the control group was 41.20%, and the inhibition rates of aseptic filtrate, 1/2 diluent and 1/4 diluent on microsclerotia germination were 78.64%, 71.36% and 60.19%, respectively. The results showed that EBS03 sterile filtrate could inhibit the germination of microsclerotia (Fig. 4).

Greenhouse experiment to determine the control effect of *B. subtilis* EBS03 on Verticillium wilt of cotton

The Verticillium wilt disease incidence of cotton seedlings in the greenhouse was investigated 15 days after inoculation with *V. dahliae*. The results of the seed soaking test showed that the diseased plant rate and disease index of the treatment group were significantly lower than those of the control group. The diseased plant rate of the treatment group and the control group was 4.17% and 31.95%, respectively, the disease index was 8.17 and 34.51, and the greenhouse control effect on cotton Verticillium wilt was 76.33%. The results of the root irrigation test showed that the diseased plant rate and disease index of cotton seedlings treated with root irrigation decreased by 12.49% and 25.94%, respectively, compared with the control group, and the control effect on cotton Verticillium wilt was 87.11%. The results showed that both the EBS03 seed soaking and root irrigation could significantly reduce the diseased plant rate and disease index of cotton seedlings and improve the control effect of cotton seedlings against Verticillium wilt (Table 2; Fig. 5).

Table 2 Control effects of different treatments of EBS03 against cotton Verticillium wilt

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

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

Comparison of emergence and growth indexes of cotton under different treatments in the greenhouse

When cotton seedlings were sown for 60 days, 15 cotton plants were randomly selected from each treatment to measure the plant height, root length, fresh matter quality, and other biomass indicators. The results showed that EBS03 soaking and root irrigation treatment can significantly improve the root length and plant height of cotton, aboveground fresh matter quality, and fresh matter quality, the increases were 35.00% and 43.39%, 78.58% and 78.08%, 69.14% and 113.33%, 69.32% and 107.22%, respectively, indicating that EBS03 has a promoting effect on the growth and development of cotton seedlings (Table 3).

Table 3 Effects of different treatments of EBS03 on cotton growth indexes

	Detection index				
Treatment	Emergence rate(%)	Root length (cm)	Plant height (cm)	Aboveground fresh material quality (g)	Quality of fresh material (g)
Seed soaking	71.53	7.56±0.23 *	23.93±0.28 *	1.37±0.01 *	1.49±0.02 *
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 *
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 ($P < 0.05$)

Field control effect of *B. subtilis* EBS03 on **cotton Verticillium wilt**

As shown in Table 4, in the field, at the 60 and 80 days of cotton sowing, the disease index of spray treatment was significantly lower than that of the control treatment, and the control effects were 42.54% and 35.65%, respectively; the control effects of fermentation liquid root irrigation treatment were 31.18% and 21.08%, respectively; the treatment of seed soaking in fermentation broth had slightly lower control of cotton Verticillium wilt, 24.98% and 17.82%, respectively. Three kinds of EBS03 treatments significantly reduced the disease index of cotton Verticillium wilt, among which the effect of EBS03 fermentation broth spray on cotton Verticillium wilt was the best.

Table 4 Effects of different treatments of EBS03 against cotton Verticillium wilt

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
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
Control	18.54±2.45	/	26.14±0.90	/
Atomization	12.51±0.18 *	42.54±0.83	16.82±0.40 *	35.65±1.63
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 ($P < 0.05$)

Determination of seedling emergence and growth indexes of cotton by seed soaking in field

The emergence rate of cotton seedlings treated with EBS03 was 67.33%, which was significantly higher than that of the control group (61.00%), indicating that strain EBS03 played a significant role in promoting the emergence of cotton seedlings. In addition, the root length, plant height, aboveground fresh matter quality, and fresh matter quality of cotton seedlings treated with strain EBS03 were not significantly different from those of the control (Table 5).

Table 5 Effect of EBS03 on seedling biomass of cotton

Treatment	Detection index				
	Emergence rate(%)	Root length(cm)	Plant height (cm)	Aboveground fresh material quality(g)	Quality of fresh material(g)
Seed soaking	67.33±7.69 *	7.58±0.36 *	19.39±0.47	34.28±0.28	38.09±0.76
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 ($P < 0.05$)

Effect of *B. subtilis* EBS03 on fiber quality and yield-related characters of cotton in the field

In the field test, seed soaking, root irrigation, and spraying could increase plant height, 30 boll seed cotton weight, and 30 boll lint cotton weight, and the differences were significant. Among them, the seed cotton weight of 30 bolls and the tare weight of 30 bolls treated by seed soaking, root irrigation and spraying increased by 11.20 g and 2.30 g, 4.44 g and 1.94 g, 16.06 g and 2.67 g, respectively, with increases of 7.42% and 3.80%, 2.62% and 2.99%, 9.20% and 4.15% (Table 6). Furthermore, EBS03fermentation liquid root irrigation and spray treatment both improved the quality of cotton fiber (Table 7).

Table 6 Effects of different treatments of EBS03 on cotton yield and yield-related traits

Treatment	Height (cm)	Number of branches	Number of bells 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 *
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 *
Control	77.87±0.74	15.33±0.35	12.07±1.27	169.33±2.05	64.93±0.90
Atomization	81.33±0.64 *	16.20±0.12 *	14.07±0.24 *	178.83±3.27 *	67.00±2.16 *
Control	70.47±0.07	13.87±0.18	9.13±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 Comparison of fiber quality under different treatments

Treatment	Average length of the upper half (mm)	Neatness index (%)	Specific strength to break ($cN•tex^{-1}$)	The marlon value	Elongation (%)
Root irrigation	30.00	86.30	30.50	5.00	6.80
Control	29.00	84.20	27.90	5.30	6.70
Atomization	29.30	86.00	31.60	4.80	6.80
Control	27.00	85.00	29.60	5.10	6.70

Detection of cotton resistance to *V. dahliae* induced by fermentation broth of strain EBS03

After the true leaves were cultured on the surface of a water agar medium for 7 days, the disease of the leaves was observed. In the EBS03 root irrigation treatment, the mycelium attachment on the leaf surface was less, while in the aseptic LB root irrigation control, there were a large number of mycelium colonization on the leaf surface, a large necrotic area, and serious leaf damage (Fig. 6).

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

The production and accumulation of reactive oxygen species in cotton leaves were determined by DAB staining. The results showed that there were more brown precipitates in cotton leaves treated with EBS03 root irrigation, but less in the control, indicating that EBS03 induced the outbreak of reactive oxygen species in cotton leaves (Fig. 7).

Callose deposition in cotton leaves induced by EBS03

Two days after inoculation with strain EBS03, callose accumulation in cotton leaves was detected. The results of the microscopic examination showed that the accumulation of callose in cotton leaves treated with EBS03 was higher than that in the control, indicating that EBS03 induced the accumulation of callose in cotton leaves (Fig. 8).

Expression of defense-related genes induced by EBS03 in cotton

The expressions of defense-related genes *POD*, *PPO*, *PAL* and *PR*10 in cotton leaves were detected by fluorescence quantitative qPCR. The results showed that. EBS03 induced the up-regulated expression of defense-related genes *POD*, *PPO*, *PAL* and *PR*10 in cotton plants. Among them, the expression of *POD* and *PAL* in the EBS03 suspension inoculated with *V. dahliae* Vd080 reached the highest level at 72 hpi, which were 6.16 and 4.37 times higher than that of the control, respectively. The expression of *PPO* at 24 hpi was the highest, and the highest expression was 6.34 times higher than that of the control (Fig. 9).

Detection of *V. dahliae* in cotton tissue by qPCR

The results of real-time quantitative PCR showed that the DNA content of *V. dahliae* in the hypocotyls of the EBS03 treatment group and the control group was significantly different at 5 days after inoculation, with 3.38 times that of the control group compared to EBS03 treatment, indicating that the EBS03 fermentation broth could inhibit the reproduction of Vd080 in plants, reduce colonization (Fig. 10).

Discussion

It is a green control strategy to use antagonistic microorganisms and their metabolites to inhibit the occurrence and harm of plant pathogens (Sehrawa et al. 2022). At present, it has been found that *B. subtilis* has an inhibitory effect on a variety of plant pathogens. In this study, through the indoor bacteriostatic test, greenhouse test and field test, it was found that the inhibition rates of EBS03 on the mycelial growth of *V. dahliae* were 70.03% and 59.00%, respectively. The inhibition rates of sporulation and microsclerotia germination were 47.16% and 70.06%, respectively. In the greenhouse test, the control effect of EBS03 root irrigation on cotton Verticillium wilt was the highest at 87.11%, and the disease-resistance system of cotton could be induced and activated by *B. subtilis* EBS03. The microbial agents based on this strain will be further studied in the future, and the biocontrol potential of this strain against cotton seedling disease, Fusarium wilt, and anthracnose will be paid more attention to.

At present, the determination of the biocontrol potential of endophytes is mostly carried out in the laboratory or greenhouse, but less in the field (Li et al. 2021). The biocontrol strains with strong antagonistic effects against pathogens were screened in the laboratory, and the control effect was unstable in the field. In the greenhouse experiment, the seed soaking method and root irrigation method were used to treat greenhouse cotton seedlings, and the control effects were 76.33%-87.11%, but the control effects in the field were 24.98%-42.54% (60 days). The reason may be that the endophyte was a

living organism, and the field environment and some abiotic factors will affect the control effect of endophytes (Wang et al. 2007). Some studies have confirmed that biocontrol bacteria can increase cotton yield, plant height, boll number per plant, fruit branch number and so on. In the greenhouse experiment, the root length, plant height, aboveground fresh matter quality, and total fresh matter quality of cotton seedlings treated with *B. subtilis* EBS03 solution were significantly higher than those of the control group. The increases were 35.00% and 43.39%, 78.58% and 78.08%, 69.14% and 113.33%, 69.32% and 107.22%, respectively. It had a good effect on promoting the growth of cotton seedlings. In the field experiment, seed soaking, root irrigation, and spray treatment in EBS03 culture solution had significant effects on the quality of 30 bolls of seed cotton and 30 bolls of lint, with increases of 7.42% and 3.80%, 2.62% and 2.98%, 9.20% and 4.15%. The results showed that *B. subtilis* EBS03 could not only control *V. dahliae* but also increase cotton yield.

The induced resistance of biocontrol bacteria needs to cooperate with competition, hyperparasitism, antagonism, plant growth promotion, and other mechanisms to play a synergistic effect (Henry et al. 2011). Reactive oxygen species play an important role in regulating plant growth and enhancing stress resistance. Callose is regarded as a physical barrier for plants to resist pathogens (Qi et al. 2018). After root irrigation with the EBS03 solution, the infection of *V. dahliae* on cotton leaves was significantly reduced, the burst of reactive oxygen species and callose accumulation in cotton leaves were increased, and the colonization of *V. dahliae* in cotton leaves was reduced. Phenylalanine aminolase (*PAL*) is a key enzyme in the synthesis of lignin, phenols, and other antibacterial substances, which is very important for the formation of a plant disease resistance system (Lv et al. 2017). Plant course-related protein *PR*10 is a kind of disease-related protein produced by plants under various biotic and abiotic stresses, which plays an important role in plant development and stress (Jannoey et al. 2017). This study found that the up-regulated expressions of *PAL*, *POD*, *PPO* and *PR*10 were induced by root irrigation with EBS03 solution, which showed broad-spectrum induced resistance. The expression of *POD* and *PAL* in the spore suspension inoculated with *V. dahliae* Vd080 reached the peak at 72 hpi, which was 6.16 times and 4.37 times higher than that of the control, while the expression of *PPO* reached the peak at 24 hpi and the highest expression was 6.34 times that of the control. *B. subtilis* EBS03 can induce plant disease resistance, but this study is only a preliminary study on the defense mechanism, and its deeper mechanism and signal transduction pathway needs to be further studied.

Conclusion

In this study, *B. subtilis* EBS03 could effectively inhibit mycelium growth, conidia production, and microsclerotia germination of *V. dahliae*. Greenhouse and field experiments showed that the application of EBS03 could reduce the incidence and disease index of Verticillium wilt, and promote the growth, development, and yield of cotton. In addition, *B. subtilis* EBS03 induced cotton leaf resistance to Verticillium wilt, and caused the outbreak of reactive oxygen species, callose accumulation, and up-regulated expression of defense genes in cotton, indicating that EBS03 activated cotton systemic disease resistance. To sum up, *B. subtilis* EBS03 has a good application prospect in the control of cotton Verticillium wilt and provides a foundation for the research and development of microbial agents.

Declarations

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Authors' contributions

Conceptualization: Zhang YL, Peng J and Zhu HQ; 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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Availability of data and materials

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

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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References

1. Acharya B, Ingram TW, Oh Y, et al. Opportunities and challenges in studies of host-pathogen interactions and management of *Verticillium dahliae* in tomatoes. Plants. 2020;9(11):1622. https://doi.org/10.3390/plants9111622.
2. Aini N, Jibril AN, Liu S, et al. Advances and prospects of genetic mapping of Verticillium wilt resistance in cotton. J Cotton Res. 2022;5(01):48–58. https://doi.org/10.1186/s42397-021-00109-0.
3. Bai HY, Feng ZL, Feng HJ, et al. Evaluation of the control effect of 48 strains of *Bacillus subtilis* on cotton Verticillium wilt. China Cotton. 2021;48(12):13–19. https://doi.org/10.11963/cc20210199.
4. Berg G, Fritze A, Roskot N, et al. Evaluation of potential biocontrol rhizobacteria from different host plants of *Verticillium dahliae* Kleb. J Appl Microbiol. 2010;91(6):963–971. https://doi.org/10.1046/j.1365-2672.2001.01462.x.
5. Bubici G, Marsico AD, D'Amico M, et al. Evaluation of Streptomyces spp. for the biological control of corky root of tomato and Verticillium wilt of eggplant. Appl Soil Ecol. 2013;72:128–134. https://doi.org/10.1016/j.apsoil.2013.07.001.
6. Chandrasekaran M, Belachew ST, Yoon E, et al. Expression of *β-1, 3-glucanas*e (*GLU*) and *phenylalanine ammonia-lyase* (*PAL*) genes and their enzymes in tomato plants induced after treatment with *Bacillus subtilis* CBR05 against *Xanthomonas campestris* pv. vesicatoria. J Gen Plant Pathol. 2017;83(1):7–13. https://doi.org/10.1007/s10327-016-0692-5.
7. Chi BJ, Zhang DM, Dong HZ, et al. Control of cotton pests and diseases by intercropping: A review. J Integr Agric. 2021;20(12):3089–3100. https://doi.org/10.1016/S2095-3119(20)63318-4.
8. China National Institute of Standardization. Determination of antifungal activity for microbial secondary metabolites-mycelial growth rate method: GB/T38480-2020. Beijing: China Standard Press; 2020.
9. Fradin EF, Thomma B. Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Mol Plant Pathol. 2010;7(2):71–86. https://doi.org/10.1111/j.1364-3703.2006.00323.x.
10. Huang W, Zhang Y, Zhou J, et al. The respiratory burst oxidase homolog protein D (GhRbohD) positively regulates the cotton resistance to *Verticillium dahliae*. Int J Mol Sci. 2021;22(23):13041. https://doi.org/10.3390/ijms222313041.
11. Henry G, Deleu M, Jourdan E, et al. The bacterial lipopeptide surfactin targets the lipid fraction of the plant plasma membrane to trigger immune-related defense responses. Cell Microbiol. 2011;13(11):1824–1837. https://doi.org/10.1111/j.1462-5822.2011.01664.x.
12. Ingram TW, Oh Y, Adhikari TB, et al. Comparative genome analyses of 18 *Verticillium dahliae* tomato isolates reveals phylogenetic and race specific signatures. Front Microbiol. 2020;11:573755. https://doi.org/10.3389/fmicb. 2020. 573755.
13. Jannoey P, Channei D, Kotcharerk J, et al. Expression analysis of genes related to rice resistance against brown planthopper, nilaparvata lugens. Rice Science. 2017;03(v.24):46–55. https://doi.org/CNKI: SUN: SDKE.0.2017-03-005.

14. Li RZ, Zhao FT, Wang ZW, et al. Study on biological characteristics of bt transgenic cotton SCRC 21 with high and stable yielding capacity. Cotton Sci. 2009;21(03):230–235 + 242. DOI:10.3969/j.issn.1002-7807.2009.03.013.
15. Li L, Gao L, Liu YH, et al. Diversity of cultivable endophytic bacteria associated with halophytes in Xinjiang of China and their plant beneficial traits. J Arid Land. 2021;13(8):790–800. https://doi.org/10.1007/s40333-021-0016-2.
16. Lv M, Kong H, Liu H, et al. Induction of phenylalanine ammonia-lyase (PAL) in insect-damaged and neighboring undamaged cotton and maize seedlings. Int J Pest Manage. 2017;63(2):166–171. https://doi.org/10.1080/09670874.2016.1255804.
17. Mitra D, Mondal R, Khoshru B, et al. Actinobacteria-enhanced plant growth, nutrient acquisition, and crop protection: Advances in soil, plant, and microbial multifactorial interactions. Pedosphere. 2022;32(1):149–170. https://doi.org/10.1016/S1002-0160(21)60042-5.
18. Qi JS, Song CP, Wang BS, et al. Reactive oxygen species signaling and stomatal movement in plant responses to drought stress and pathogen attack. J Integr Plant Biol. 2018;60(9):805–826. https://doi.org/10.1111/jipb.12654.
19. Sehrawat A, Sindhu SS, Glick BR. Hydrogen cyanide production by soil bacteria: Biological control of pests and promotion of plant growth in sustainable agriculture. Pedosphere. 2022;32(1):15–38. https://doi.org/10.1016/S1002-0160(21)60058-9.
20. Sawada H, Fujikawa T, Horita H. *Pseudomonas brassicae* sp. nov, a pathogen causing head rot of broccoli in Japan. Int J Syst Evol MicroBiol. 2020;70(10):5319–5329. https://doi.org/10.1099/ijsem.0.004412.
21. Sahebani N, Gholamrezaee N. The biocontrol potential of Pseudomonas fluorescens CHA0 against root knot nematode (Meloidogyne javanica) is dependent on the plant species. Biol Control. 2021;152:104445. https://doi.org/10.1016/j.biocontrol.2020.104445.
22. Sun C, Shao YQ, Vahabi K, et al. The beneficial fungus *Piriformospora indica* protects *Arabidopsis* from *Verticillium dahliae* infection by downregulation plant defense responses. BMC Plant Biol. 2014;14:268. https://doi.org/10.1186/s12870-014-0268-5.
23. Varo A, Raya-Ortega MC, Trapero A. Selection and evaluation of micro-organisms for biocontrol of *Verticillium dahliae* in olive. J Appl Microbiol. 2016;121. https://doi.org/10.1111/jam.13199.
24. Wei F, Feng H, Zhang D, et al. Composition of rhizosphere microbial communities associated with healthy and Verticillium wilt diseased cotton plants. Front Microbiol. 2021;12:618169. https://doi.org/10.3389/fmicb.2021.618169.
25. Wang JJ, Zhao DY, Liu YG, et al. Antagonism against Beauveria bassiana by lipopeptide metabolites produced by entophyte Bacillus amyloliquefaciens strain SWB16. Acta Microbiol Sinica. 2014;54(07):778–785. https://doi.org/10.13343/j.cnki.wsxb.2014.07.008.
26. Wang LT, Lee FL, Tai CJ, et al. Comparison of gyrB gene sequences, 16S rRNA gene sequences and DNA-DNA hybridization in the *Bacillus subtilis* group. Int J Syst Evol MicroBiol. 2007;57(8):1846–1850. https://doi.org/10.1099/ijs.0.64685-0.

27. Yuan Y, Feng H, Wang L, et al. Potential of endophytic fungi isolated from cotton roots for biological control against Verticillium wilt disease. PLoS ONE. 2017;12(1):e0170557. https://doi.org/10.1371/journal.pone.0170557.
28. Zhao SS, Zhang YY, Yan W, et al. Chaetomium globosum CDW7, a potential biological control strain and its antifungal metabolites. Microbiol Lett. 2017;364(3):fnw287. https://doi.org/10.1093/femsle/fnw287.
29. Zhao Y, Selvaraj JN, Xing F, et al. Antagonistic action of *Bacillus subtilis* strain SG6 on Fusarium graminearum. PLoS ONE. 2014;9(3):e92486. https://doi.org/10.1371/journal.pone.00924864.
30. Zhang YL, Zhou JL, Zhao LH, et al. A review of the pathogenicity mechanism of *Verticillium dahliae* in cotton. J Cotton Res. 2022;5(1):1–13. https://doi.org/10.1186/s42397-021-00111-6.
31. Zhang YL, Li ZF, Feng ZL, et al. Isolation and functional analysis of the pathogenicity-related gene *VdPR3* from *Verticillium dahliae* on cotton. Current Genetics. 2015;61(4):555–566. https://doi.org/10.1007/s00294-015-0476-z.
32. Zhang P, Wang JM, Yuan XH, et al. Characters selection and assessment for spathiphyllum DUS testing guideline. Agricultural Biotechnol. 2021;10(05):21–25 + 48. https://doi.org/10.19759/j.cnki.2164-4993.2021.05.007.
33. Zhu HQ, Feng ZL, Li ZF, et al. Identification of cotton varieties (lines) resistance to Verticillium wilt by quantitative dipping method of vermiculite sandy bottomless paper pot. China Cotton. 2010;37(12):15–17. https://doi.org/10.3969/j.issn.1000-632X.2010.12.006.
34. Zhao LH, Feng ZL, Li ZF, et al. Development of an improved standard for identifying and evaluating Verticillium wilt resistance in cotton. Cotton Sci. 2017;29(01):50–58. https://doi.org/10.11963/issn.1002-7807.201701006.
35. Zhang Y, Zhao LH, Feng ZL, et al. The role of a new compound micronutrient multifunctional fertilizer against *Verticillium dahliae* on Cotton. Pathogens. 2021;10(1):81. https://doi.org/10.3390/pathogens10010081.
36. Zhang Y, Feng ZL, Feng HJ, et al. Control effect of endophytic fungus *Chaetomium globosum* CEF-082 against Verticillium wilt in *Gossypium hirsutum*. Acta Phytopathologica Sinica. 2016;46(5):697–706. https://doi.org/10.13926/j.cnki.apps.2016.05.015.

Figures

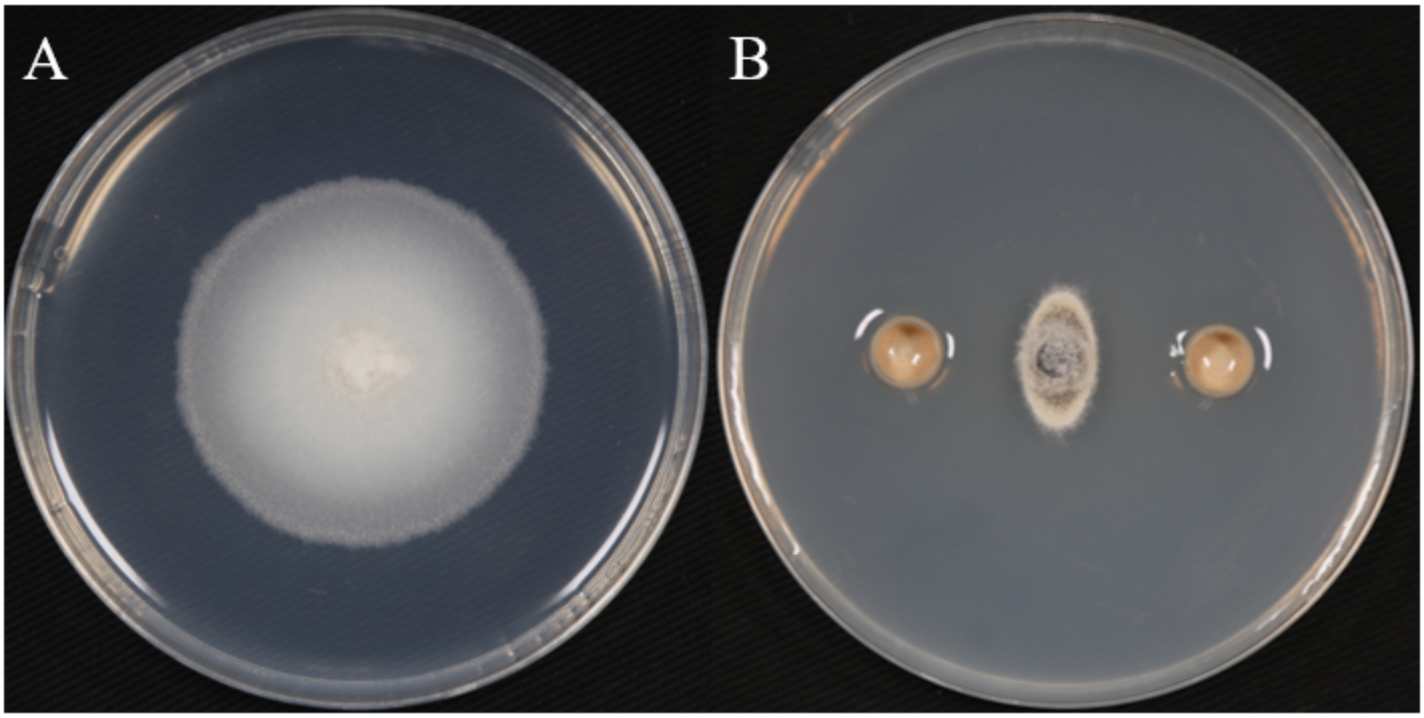

Figure 1

Inhibitory effect of EBS03 non-volatile metabolites on the colony growth of *V. dahliae*

A: control; B: treated by EBS03

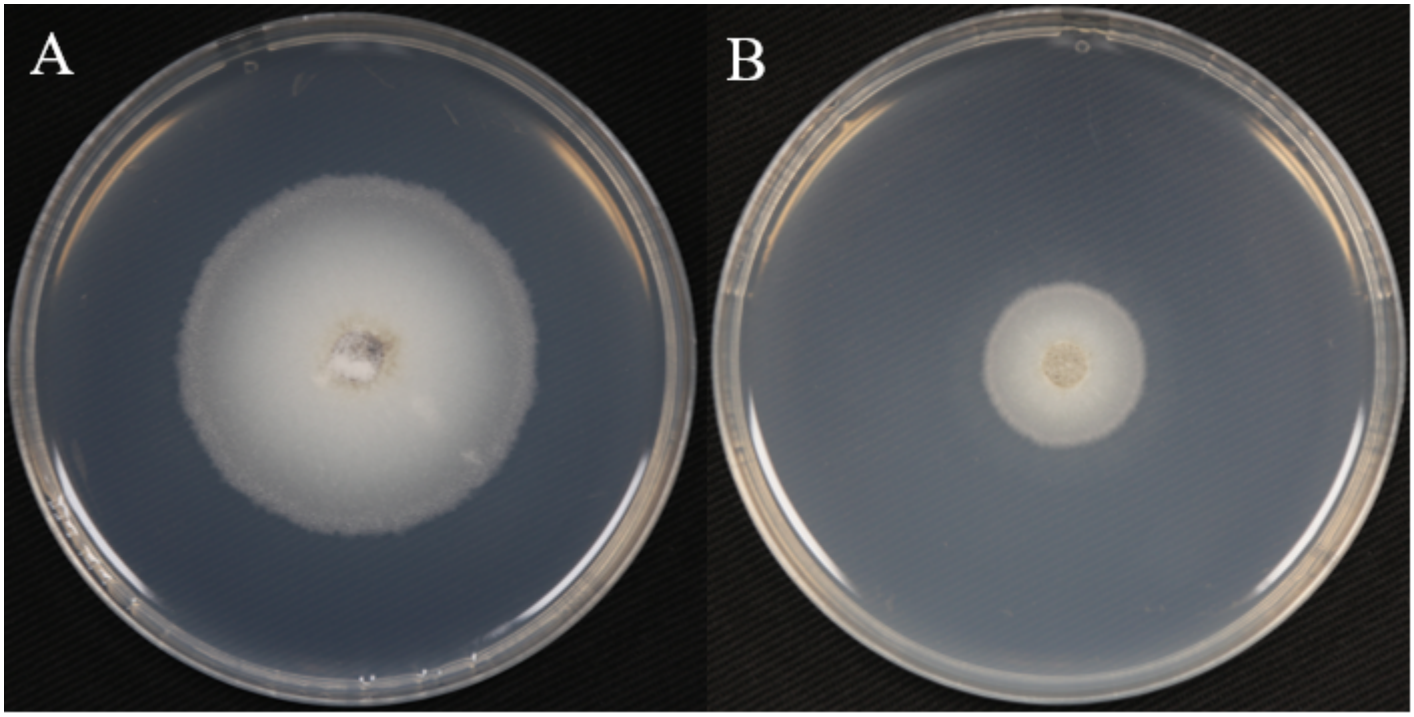

Figure 2

Effects of EBS03 volatile metabolites on the colony growth of *V. dahliae*

A: control: B: treated by EBS03

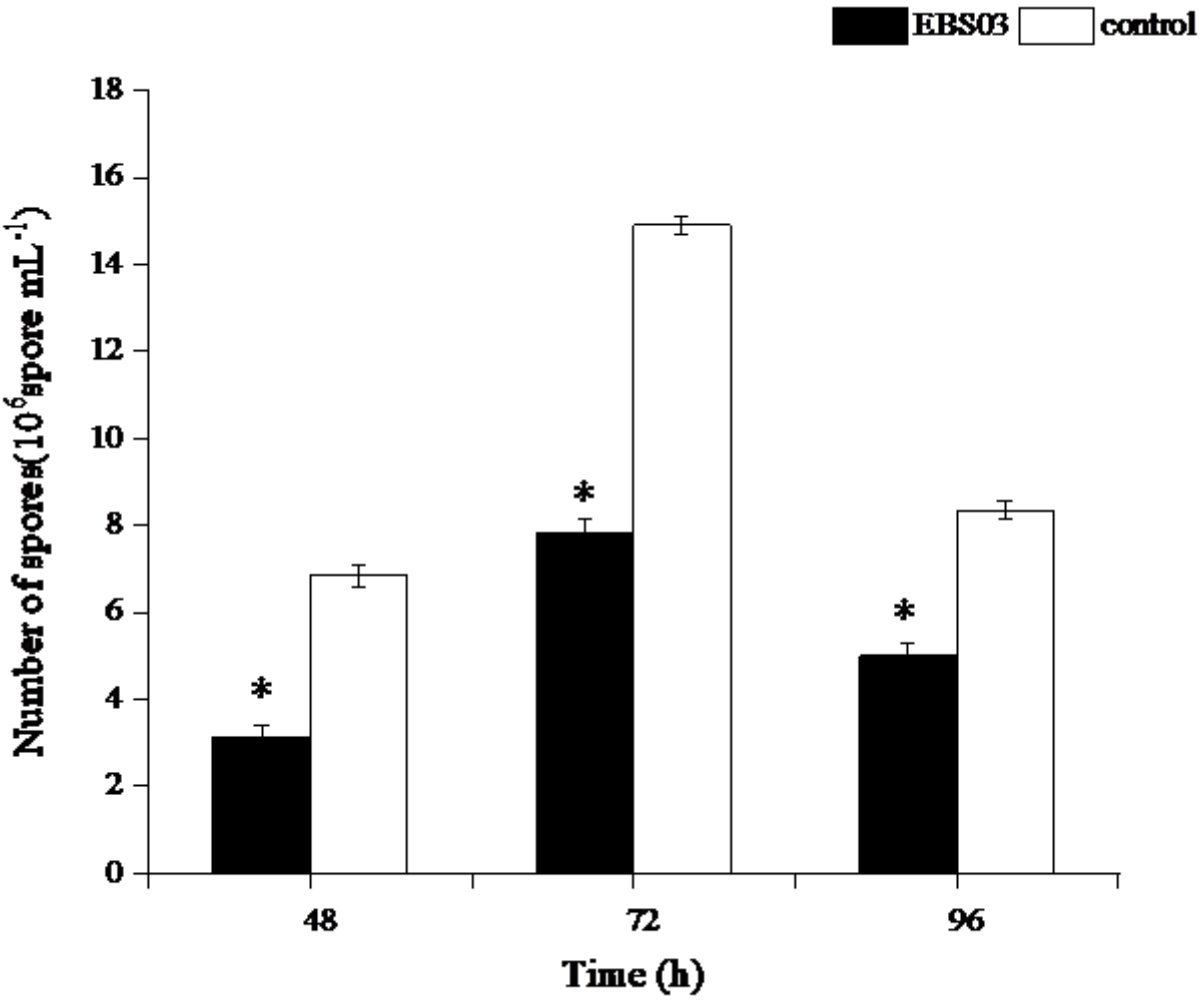

Figure 3

Effect of EBS03 on spore production of *V. dahliae*

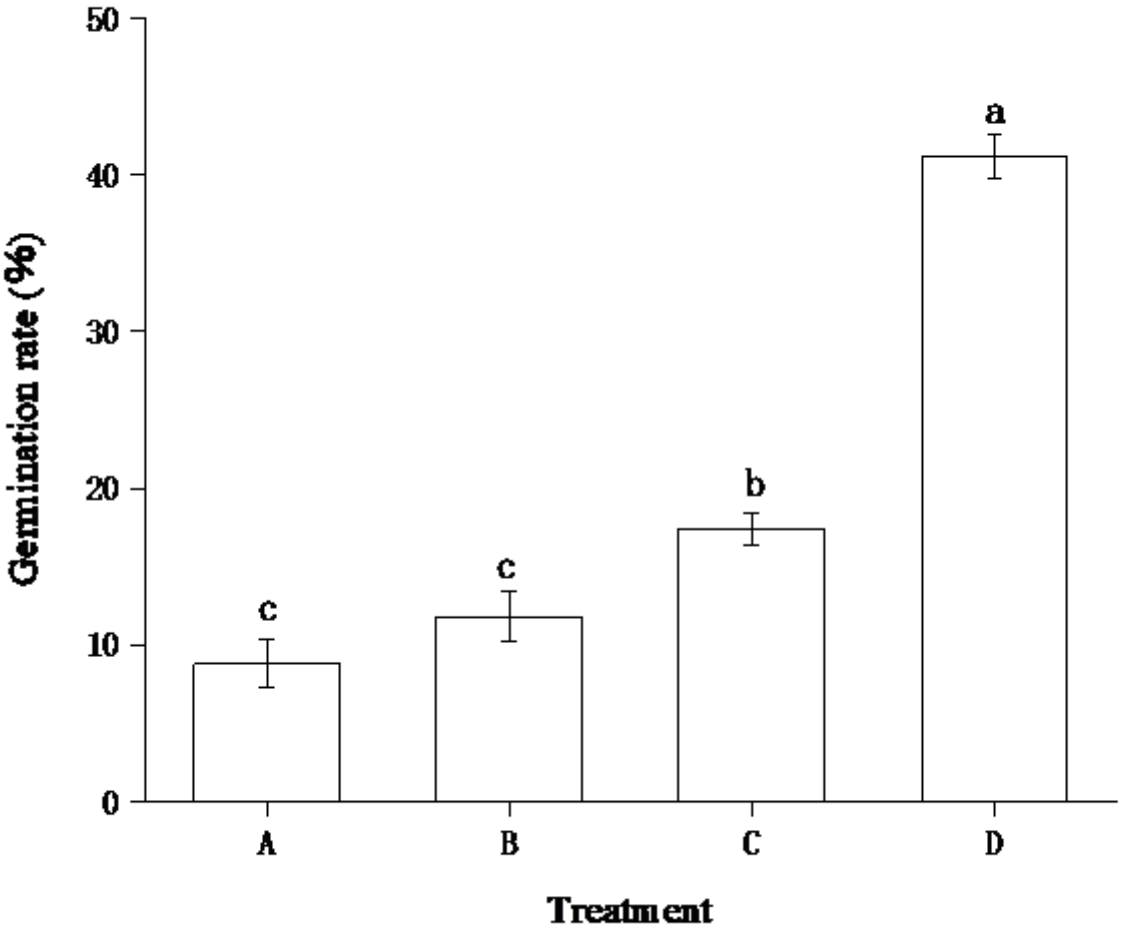

Figure 4

Effects of EBS03 on sclerotium germination

A: Sterile filtrate stock solution; B: 1/2dilution; C: 1/4dilution; D: LB medium

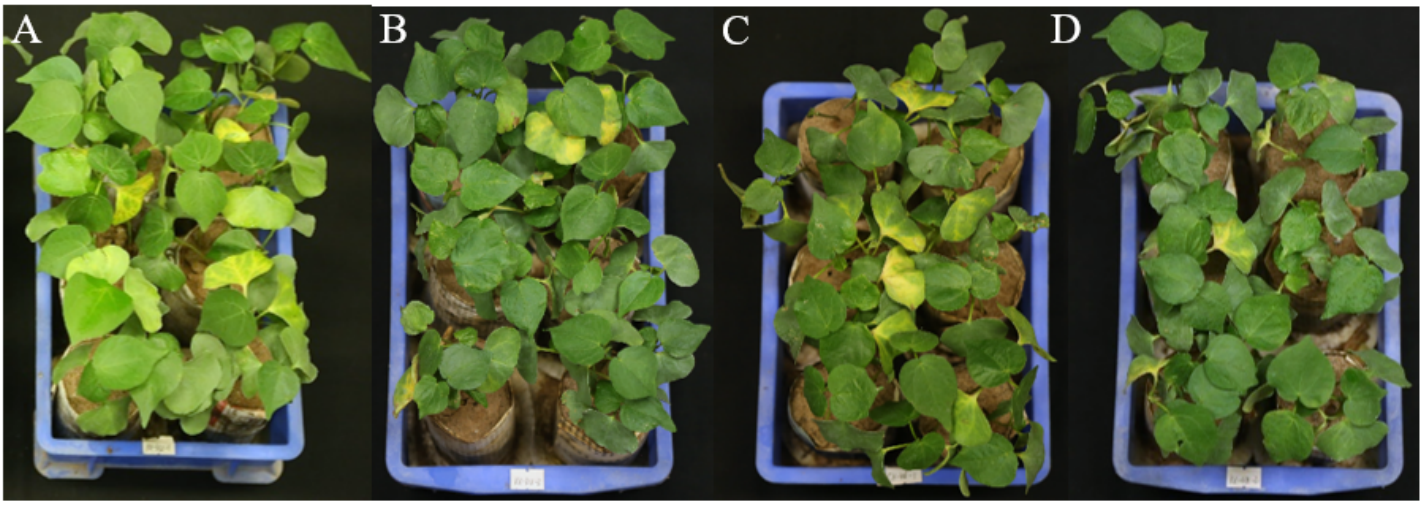

Figure 5

The incidence of cotton seedlings two weeks after EBS03 inoculation

A: the control of EBS03 seed soaking treatment; B: EBS03 seed soaking treatment; C: the control of EBS03 root irrigation treatment; D: EBS03 root irrigation treatment

Figure 6

EBS03 enhanced cotton leaves against *V. dahliae*

A: control; B: treated by EBS03

Figure 7

EBS03 triggered ROS in cotton

A: control; B: treated by EBS03

Figure 8

EBS03 induces callose accumulation in cotton leaves

A: control; B: treated by EBS03

Figure 9

EBS03 induces the expression of defense-related genes

A-E: Expression of *POD*、*PPO*、*PAL*、*PR*10; control means cotton was first treated with LB medium, and then inoculated with Vd080; EBS03 means cotton was first treated with EBS03 culture solution and then inoculated with Vd080. * indicate a significant difference at the level of $P \leq 0.05$.

Figure 10

DNA content of *V. dahliae* in hypocotyls of cotton seedlings