

Discovery of Endophytic Strains With Excellent Antagonism to *Colletotrichum Scovillei* in Sweet Pepper and Study on Their Biological Functions

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Research Article

Keywords: Antagonistic Strains, Screening, Identification, Biological Function Determination, Potted Control

Posted Date: July 19th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-708736/v1>

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Abstract

Anthracnose caused by *Colletotrichum* spp. is a well-known disease that causes severe losses in pepper production which is used as a spice for thousands of households in many parts of China. With the biological control properties of endophytic strains have been developed and used have been of great importance in the prevention and control of anthracnose and environmental protection. Therefore, to control the pathogen *Colletotrichum scovillei* more safely, 58 endophytic strains were isolated from pepper leaves in this experiment. Plate resistance method was used to screen the antagonistic strains of *C. scovillei*, and it was found that the inhibition rate of 25 antagonistic strains against *C. scovillei* was greater than 60%, and the inhibitory rates of L1-7 and L3-5 against it were 79% and 80%, respectively. They were identified as *Bacillus amyloliquefaciens* and *Bacillus velezensis* by culture and morphological identification, combined with 16S rDNA and *gyrB* gene sequence analysis. The two antagonistic endophytic bacteria also had the ability to fix nitrogen and secrete IAA, and all had high salt tolerance. Controlled pot experiments in the laboratory showed that L1-7 and L3-5 had a good control effect against *C. scovillei*, with the control efficiency reaching 80.64% and 73.39%, respectively. Thus, *B. amyloliquefaciens* (L1-7) and *B. velezensis* (L3-5) could be useful as biological control agents to protect peppers from anthracnose disease caused by *C. scovillei*. The results of this test provide a basis for the development of pepper endophytic bacterial resources, and also bacterial resources for the biological control of *C. scovillei*.

Introduction

Endophytes include primarily bacteria, fungi and actinomycetes. They are a large group of microorganisms that survive in healthy living plant tissue without causing obvious pathological changes in the host plant (Hallmann et al. 1997). They are not only widely distributed in plants, but also have formed a mutually beneficial, symbiotic and interdependent relationship with plants in the long-term evolutionary process, which can promote plant growth, endow plants with stress resistance, pest resistance and antagonistic functions (Zhao et al. 2010; Liu et al. 2016b; Ding et al. 2017), so plant endophytes have excellent biological control potential. At present, research on endophytes focuses on promoting plant growth, acting as a nutrient and inhibiting the growth of pathogenic microorganisms (Ahmad et al. 2008; Mnasri et al. 2016; Dilfuza et al. 2017; Hassan 2017). Pepper anthracnose causes major production losses worldwide where pepper plants are grown (Silva et al. 2019). In addition to affecting peppers, these fungi also cause diseases found in a wide range of vegetables, fruits and other crops (Rai et al. 2009). Therefore, prevention and treatment of pepper anthracnose caused by *Colletotrichum* is extremely urgent. Currently, various methods are used to control different plant diseases. Among them, chemical control is currently the most effective method for controlling plant diseases (Hirooka and Ishii 2013). However, the use of fungicides can pose a serious threat to the environment and cause harmful side effects to humans. Biocontrol of plant diseases is the suppression of populations of plant pathogens by microbial antagonists or production of antimicrobials. Therefore, alternatives to microbial antagonist fungicides have been selected as biocontrol agents (Khl et al. 2019; Prisana et al. 2019).

Furthermore, rational use of endophytic strains to control pathogens is the general trend (Bhattacharya et al. 2019). On this basis, many endophytic strains have been isolated and shown to be able to control a variety of pathogens, including antagonistic bacteria, antagonistic fungi and antagonistic actinomycetes (Jiang and Song 2014). For example, studies have shown that the endophytic fungus, *Ramichloridium* sp. tested in the detached leaflet of açai palms (*Euterpe precatoria* Mart.) showed greater abilities in controlling anthracnose (Peters et al. 2020). The endophytic population of *Pseudomonas* isolated from tomato inhibited the mycelial growth of *Pythium aphanidermatum* and *Pythium ultimum* (Gravel et al. 2005). The four yeast species *Pichia guillier-mondii*, *Candida musae*, *Issatchenkia orientalis* and *Candida quercitrusa* have been isolated from Thai fruits and vegetables. They can antagonistically control *Colletotrichum capsici*, and the inhibition rates are 66.4%, 76.6%, 83.1% and 93.3%, respectively (Chanchaichaovivat et al. 2007; Chanchaichaovivat et al. 2008). Various endophytic strains have been used countless times in biocontrol, but endophytic bacteria are the most commonly used (Asghari et al. 2019). In the isolation of endophytic bacteria, according to existing article, the dominant strains of endophytic bacteria were *Bacillus*, *Pseudomonas* and *Streptomyces* (El-Deeb et al. 2013; Liu et al. 2016c). The endophytic bacterium *Bacillus pumilus* caused the plants to produce a large amount of deposited tannins and phenols, which thickened the cell wall, and pathogens were prevented from invading between the outer layer and the cork layer (Benhamou et al. 1996). 2, 4-Diacetyl-phloroglucinol was a phenolic compound produced by *Pseudomonas*, that has anti-viral, anti-fungal and preventive activity against other bacteria (Hao 2010). *Streptomyces griseocarneus* R132 inhibited the growth of plant pathogens with a range of $57.24 \pm 4.54\%$ (*Fusarium oxysporum* 46.7) to $73.93 \pm$

3.71% (*Botryosphaeria dothidea* CFMAC3), which apparently controlled the development of anthracnose symptoms caused by *Colletotrichum gloeosporioides* (Liotti et al. 2019). Although there have been many studies on antagonistic endophytic bacteria, they have not been reported with biological control function against *C. scovillei*.

Therefore, in this experiment the endophytic bacteria were isolated from healthy pepper leaves, the strains with good antagonistic effects on *C. scovillei* were screened and the identification and biological function determination were carried out which lays the foundation for the biological control of pepper anthracnose.

Materials And Methods

Tested materials

Endophytic strains: endophytic strains were isolated from the healthy leaves of sweet pepper (Pepper plants are grown in the biocontrol engineering laboratory of crop diseases and pests of Gansu Province).

The medium used in the experiment: Nutrient Agar (NA) medium, Potato Dextrose Agar (PDA) medium (Fang 1998); King's medium, Pikovaskaia's (PKO) medium, Mehkha medium, Ashby medium (Cui et al. 2016; Wei et al. 2018b).

Standard colorimetric solution PC and S2: PC (First, 12 g FeCl_3 was dissolved in 300 mL sterile distilled water, then slowly added 429.7 mL H_2SO_4 with concentration of 98% and stirred continuously, waiting for the mixture to cool down and holding it to 1 L. The determination value of the colorimetric solution was 0.3 ~ 20 mg/L); S2 (First, 4.5 g FeCl_3 was dissolved in 300 mL sterile distilled water, then slowly added 587.4 mL H_2SO_4 with concentration of 98% and stirred continuously, waiting for the mixture to cool down and holding it to 1 L. The determination value of the colorimetric solution was 5 ~ 200 mg/L).

Fungal pathogen: *C. scovillei* designated LC8 was obtained from the Biocontrol Engineering Laboratory of crop diseases and pests, Gansu agricultural university, China. The fungal pathogen was isolated from sweet pepper (*Capsicum annuum*) leaves with anthracnose and grown on potato dextrose agar (PDA) at 4°C.

Isolation of endophytic bacteria

In this study, the traditional method of tissue separation (Vetrivelkai et al. 2010) was used to isolate the endophytic strain symbiosis on sweet pepper (*Capsicum annuum*) leaves. The pepper leaves were washed with sterile distilled water, air-dried and 2 g of the dried leaves were weighed. Then, the surface of the pepper leaves was disinfected with 0.1% HgCl_2 solution for 30 s, and rinsed 5 times with sterile distilled water. The surface of the pepper leaves was then disinfected again with 75% ethanol for 45 s, washed with sterile distilled water for 5 times. We used sterile filter paper to absorb excess water and ground the leaves in a sterile mortar, and stopped grinding after the leaf juice was formed and 0.1 mL of the leaf juice was extracted and placed in a centrifuge tube containing 9.9 mL of sterile distilled water to dilute the tissue fluid 10 times, followed it was then diluted to concentrations of 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} per mL using the aforementioned method, respectively. Next, 0.1 mL of tissue fluid was separately aspirated from the 4 tissue fluids of different concentrations obtained by the previous dilution and applied to the NA medium. Repeat each treatment three times. All petri dishes were cultured at 28°C for 3 days. After the leaves were disinfected with 75% ethanol, the 5th washing water was used as control. Any colony growth in the control was observed to determine whether sterilization of the leaf surface was complete, when the control group had no colony growth, the leaf surface was completely disinfected. After confirming that the sterilization was complete, strains in the remaining petri dishes were classified and purified, and stored at 4°C for future use.

Screening of antagonistic strains against LC8 (*C. scovillei*)

We studied the antagonistic strains to *C. scovillei* by the plate confrontation method (Glickmann and Dessaux 1995). The isolated endophytic strains were activated which cultured for 2 days on NA medium. After placing a 5 mm diameter mycelial disc of LC8 in the center of the PDA medium, then the endophytic strains were inoculated 2.5 cm around the pathogen. Each treatment had four replicates and the control had no inoculate endophytic strains. They were placed in a 25°C constant temperature incubator. The colony diameter of the control was measured after it had grown in the petri dishes for 7 d, and the colony diameter of the

pathogen inoculated with the endophytic strains was measured at the same time. Finally, the anti-pathogen rate was calculated using the following formula.

$$\text{Inhibition rate (\%)} = \frac{\left(\frac{\text{Colony diameter of Control}}{-\text{Colony diameter of inhibited fungus}} \right)}{\text{Colony diameter of Control}} \times 100\%$$

Morphological identification of antagonistic strains

Antagonistic strains were inoculated onto NA medium, and cultured at 28°C for 18–24 h before being subjected to Gram staining to observe bacterial morphology, measured length and width, and photographed with a microscope.

The antagonistic strains were cultured at 28°C for 72 hours to examine the colony morphology.

Molecular identification of antagonistic strains

We extracted endophytic strains DNA according to Tiangen kit (Ezup column genomic DNA extraction kit) and stored it at -20°C for future use.

PCR amplification was performed using the universal primer sets (27F: 5'-AGA GTT TGA TCM TGG CTC AG-3', 1492R: 5'-TAC GGY TAC CTT GTT ACG ACT T-3') and *gyrB* specific primer sets (UP-1: 5'-GAA GTC ATC ATG ACC GTT CTG CAY GCN GGN GGN AAR TTY GA-3', UP-2r: 5'-AGC AGG GTA CCG ATG TGC GAG CCR TCN ACR TCN GCR TCN GTC AT-3') (Morris et al. 2008; Bouaoud et al. 2018). The PCR amplification of both universal primer and specific primer were performed in a 50 µL mixture containing 25 µL PCR Mix (2×), 2 µL of each primer (10 µmol/L), 2 µL template DNA, and 19 µL ddH₂O, and the PCR reaction conditions of the universal primer were pre-denaturation at 94°C for 4 min, denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 90 s, 30 cycles and a final extension at 72°C for 10 min. PCR reaction conditions of the specific primer were pre-denaturation at 94°C for 4 min, denaturation at 94°C for 1 min, annealing at 57°C for 1 min, extension at 72°C for 70 s, 35 cycles and a final extension at 72°C for 10 min, stored the amplified product at 4°C after the reaction. PCR amplification was detected by electrophoresis on a 1% agarose gel. The amplified products were sent to Qingke Biology Company in Xi'an City, Shanxi Province, China for sequencing. The BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) algorithm was used with our gene sequences for approximate identification against the NCBI sequence database. The software MEGA v. 7 was used to build a distance tree with the neighbour-joining (NJ) algorithm.

Biological function determination of antagonistic strains

Determination of nitrogen fixation ability

Endophytic antagonist strains were inoculated into NA liquid medium, then placed on shaking cultivation for 24 h at 28°C, 180 r·min⁻¹. Subsequently 0.1 mL of the suspension strain was streaked into nitrogen-free Ashby medium and in a triangle flask of containing 100 mL liquid. Finally, an equal volume of sterile distilled water was inoculated into the medium to serve as control, using three replicates for each treatment. The petri dishes and triangular flasks were placed in an incubator at 28°C and shaker (28°C, 120 r·min⁻¹) for growth respectively and observed after 7 days. If there were colonies on the Ashby medium or the liquid medium became turbid, it means that it has ability to fix nitrogen (Cui et al. 2016).

Determination of phosphorus solubilizing capacity

Activated endophytic antagonistic strains were coated in PKO medium (inorganic phosphorus) and Mehknha medium (organic phosphorus), and phosphorus solubilization ability was observed after 14 days of cultivation at 28°C. Three replicates were used for each treatment. Phosphorus solubilization capacity was calculated using the following formula (Cui et al. 2016).

$$\text{Phosphorus solubilizing capacity} = \frac{(\text{Phosphorus} - \text{Solubilizing ring width})}{\text{Colony diameter}}$$

Determination of IAA production capacity

Determination of IAA content by the qualitative method: the colorimetric method of Salkowski was used to measure IAA (Dong and Cai 2001), 0.1 mL of the suspension liquid of the antagonistic endophytic strain in 100 mL King's medium which was culture without tryptophan or with 100 mg·L⁻¹ tryptophan was inoculated, and the same volume of sterile distilled water was added as a control, and they were set up 28°C, 120 r·min⁻¹ constant temperature, and the culture was shaken for 12 d. Then, 50 µL of culture liquid was withdrawn from each King's medium and added to a 1.5 mL centrifuge tube mixed with an equal volume of standard colorimetric solution PC and allowed to stand for 15 min at room temperature to observe the color reaction. Its in order to determine if the antagonistic endophyte has the function of secretion IAA. In the same way, S2 was used as a standard colorimetric solution to confirm whether the antagonists secrete IAA. Three replicates were used for each treatment, the color turns red to show that they can secrete IAA.

Determination of IAA content by the quantitative method: the suspension liquid of the antagonistic endophytic strain was cultured in 100mL King's medium for 12 days, and centrifuged at 4°C, 10000 r·min⁻¹ for 10 min. Then, 4 mL of the supernatant was taken after centrifugation and placed in a 10 mL centrifuge tube mixed with the same volume of standard colorimetric solution PC and placed in a dark environment for 30 min. Repeat each treatment 3 times. The absorbance value of the mixture was measured at a wavelength of 530 nm. The same volume of sterile distilled water was added to the centrifuge tube, which served as a blank control, and treated the same manner as the antagonistic endophytic strain. 4 mL of the PC colorimetric solution was added to a centrifuge tube containing the same volume of sterile distilled water. This solution was used to set the spectrophotometer to zero and calculate the amount of IAA secreted by the endophytic strain using the standard curve.

Making of standard curve: Two groups of IAA standard solutions with different concentrations were prepared by using IAA standard, each group contained seven different concentrations of IAA standard solutions (Group 1: 2.5, 5, 7.5, 10, 12.5, 15, 17.5 µg/mL; Group 2: 25, 50, 75, 100, 125, 150, 175 µg/mL). In the first group, 4 mL of each standard solution was sucked into a 10 mL centrifuge tube and added with PC colorimetric solution of the same volume, and the mixture was exposed to total darkness for 30 min, the absorbance value was measured at 530 nm by photometer and recorded. The second group of IAA standard solution was selected to make the standard curve of S2 colorimetric solution, and the data were collected by the method mentioned above. All data were processed in the Microsoft Excel and the standard graph were generated.

Determination of salt tolerance

The antagonistic endophytic strain study was inoculated into 100 mL NB culture medium containing 2%, 5%, 10%, 15%, 20% and 25% NaCl, and the NB culture medium without NaCl was used as control. Then the culture medium was placed on a shaker at a constant temperature of 28°C for 24 h, and each treatment was repeated three times. The absorbance at 600 nm was measured with a spectrophotometer, to determine the salt tolerance of endophytic antagonistic strains.

Pot control effect of the antagonistic strains

Healthy pepper seeds were wrapped in gauze and placed in a water bath at 55°C for 10 min. then at 30°C for 8 h. The seeds were then placed in a petri dish and cultured under illumination in a incubator at 25°C. Five uniformly germinated seeds were selected and planted in one pot (diameter: 13 cm)(Cui et al. 2019). Five had been germinated seeds planted in one pot. When the 10th leaf of pepper grew, the 9 pots were inoculated with the pathogen(LC8) and two types of control were set, one is the control was not inoculated with the pathogen and the other is control was inoculated with the pathogen but not sprayed with fermentation broth(The antagonistic endophytes were incubated in 50 mL NB medium for 48 h). Repeat each treatment 3 times. All potted plants for the experiment were placed in an incubator with constant temperature(25°C) and humidity(80%) to ensure that the peppers were infected by emerged pathogenic fungi. After 48 h, they were taken out from the incubator and placed indoors. After 24 h, we sprayed 6 pots (Spray 50 mL per pot) of the peppers inoculated with pathogenic fungi with fermentation broth including L1-7 and L3-5 were sprayed on 3 pots of peppers. Finally, the disease condition of each potted pepper was continuously observed, and the disease index and control effect were calculated according to the grading standard of pepper anthracnose disease condition (Table.1) and the following formula (Wheeler and Kent 1969; Fang 1998).

$$\text{Disease index} = \frac{\sum(\text{Diseased plants at all levels} \times \text{Grade of disease})}{\text{Total number of investigated plants} \times \text{Maximum disease grade}} \times 100$$

$$\text{Control efficiency}(\%) = \frac{\text{Control disease index} \times \text{Treatment disease index}}{\text{Control disease index}} \times 100\%$$

Statistical analysis

The data were analyzed by variance analysis. The software used for this task was IBM SPSS Statistics 25. Least-significant difference (LSD) method was used to test the significance of the difference ($P < 0.05$). The experimental data were counted and calculated by Microsoft Excel to clarify the function and fungistatic effect of endophytic strains.

Results

Isolation of antagonistic strains

According to the characteristics of colony size, morphology and color, 58 endophytic bacteria were isolated from pepper leaves, numbered L1-7, L3-5, L5-3, L6-6, L7-5, L7-6, L7-7, L7-9, L7-10 and L7-13, etc. After 72 h of control culture, there was no colony growth on the NA medium, indicating that the pepper leaves were thoroughly disinfected, and the isolated strains were all endophytic strains. The isolated endophytic strains were stored in the tube at 4°C.

Determination of antagonistic ability

Through our research, 58 endophytic strains were preliminarily investigated for their antagonistic ability against *C. scovillei*. Among them, 33 endophytic strains were found to growth inhibition rate of *C. scovillei* less than 60% and 15 endophytic strains were found to inhibition rate between 60% ~ 70%, and 8 endophytic strains were inhibition rate against the pathogen from 70–80%, and only 2 endophytic strains were inhibition rate above 80%. In order to determine the anti-pathogen stability of the antagonistic strains, we performed the second anti-pathogen test with the antagonistic endophytic strains that had a fungistatic rate of more than 70% in the first test. And it showed that the inhibition rates of strains L1-7 and L3-5 against *C. scovillei* again reached more than 79% (Fig. 1). The inhibition rate of L3-5 on *C. scovillei* reached 80% in two screening tests. The results of two screenings showed that L1-7 and L3-5 had stable and long-lasting inhibitory effect against *C. scovillei*. Whereas, the pathogenic fungi inhibited by L1-7 formed milky white villi on the PDA medium, indicating that L1-7 had an inhibitory effect at the beginning of the growth of the pathogenic fungi, which prevented the normal growth of mycelia in the later growth period. However, the color of the pathogenic fungi inhibited by L3-5 were gray-green, indicating that it did not affect the growth cycle of mycelia, but it has a significant inhibitory effect on the normal growth of mycelia. The reason for this conclusion, it was because the mycelial villi of *C. scovillei* were milky white in the early stage, which changes to grayish green in later growth stages. Therefore, the functions of these two antagonistic endophytic strains were studied in detail.

Identification of morphological characteristics

The colony of L1-7 was milky white, forming a round and nearly round colony shape. The surface of the colony in the early stage was smooth and opaque, in the later stage of growth the edge of the colony was distinctly serrated and wrinkled. There was a colorless transparent halo around the colony. The color of the colony of L3-5 was pale yellow, the shape was irregularly round, the surface was desiccated, wrinkled, dull, and the margins were irregular. L1-7 and L3-5 are both Gram-positive bacteria, rod-shaped, the size of L1-7 was $0.71 \times 2.25 \mu\text{m} \times 0.20 \times 0.40 \mu\text{m}$, the size of L3-5 was $0.86 \times 2.23 \mu\text{m} \times 0.24 \times 0.51 \mu\text{m}$ (Fig. 2).

Gene sequence analysis

Comparison of the 16S rDNA gene sequence obtained by sequencing with other sequences in the GenBank database revealed that the two antagonistic endophytic bacteria belonged to *Bacillus*. These included L1-7 and *B. amyloliquefaciens* (HM107806), which reached up to 100% similarity in the phylogenetic tree and were initially identified as *B. amyloliquefaciens*. L3-5 and *B. velezensis* (MF192765) assembled together in the phylogenetic tree with a similarity level that also reached 100% (Figs. 3 and 4), it was tentatively identified as *B. velezensis*. The universal primer (27F, 1492R) was used to amplify L1-7 and L3-5, and the DNA fragments obtained were 1433 bp and 1401 bp long respectively. L1-7 and L3-5 were deposited in GenBank (MW672320 for L1-7, MW672321 for L3-5).

Using the *Bacillus*-specific primers gyrB the antagonistic endophytic strains L1-7 and L3-5 were identified by amplification and sequencing, and compared in the GenBank database, L1-7 and L3-5 are grouped in the phylogenetic tree with *B. amyloliquefaciens* (MT793724) and *B. velezensis* (JX014631) respectively and their similarity reached 99% and 100% (Figs. 3 and 4). Thus by combining culture characteristics, morphological characteristics and the results of 16S rDNA gene sequence analysis L1-7 was identified as *B. amyloliquefaciens*, and L3-5 was identified as *B. velezensis*. The specific primer (UP-1, UP-2r) was used to amplify L1-7 and L3-5, and DNA fragments of 1195 bp and 1198 bp in length were obtained respectively. The gyrB ID of L1-7 and L3-5 were deposited in GenBank (MZ209100 for L1-7, MZ209101 for L3-5).

Biological function test

According to the biological function test, both strains L1-7 and L3-5 had no phosphorus- solubilizing ability, but both had nitrogen fixation and fungistatic function. The inhibition efficiency of L1-7 against *C. scovillei* reached 79%, and inhibition efficiency of L3-5 against *C. scovillei* reached 80%. Using the qualitative and quantitative methods, it was found that both L1-7 and L3-5 had the ability to secrete IAA, and whether the culture medium contained tryptophan or not did not affect the yield of IAA. The biological function test showed that strains L1-7 and L3-5 had multiple functions and possessed high potential for disease prevention and growth promotion (Table. 2).

Determination of the salt tolerance

Our researched suggests that both L1-7 and L3-5 had high salt tolerance. The most suitable salt concentration for the growth of strain L1-7 was NB medium containing 2% salt solution, which was significantly different from other treatments ($P < 0.05$). For the growth of L3-5, the most suitable salt concentration was 5%, which was 2.5 times that of L1-7, indicating that strain L3-5 was easier to adapt to the high salt range (Fig. 5).

Potted control effect of antagonistic strains

The leaves of a (spraying L1-7) and b (spraying L3-5) were the experimental group that had been sprayed with fermentation broth. They were compared with the leaves (c) which, without any treatment after inoculation with the pathogen, did not appear to have typical disease spots caused by *C. scovillei*(Fig. 6). Based on the statistics of the infestation it was found that the control effects of L1-7 and L3-5 on *C. scovillei* were 80.64% and 73.39%, respectively (Table. 3). Therefore, L1-7 and L3-5 played an important role in disease control. In addition, compared with leaves (d) that were not inoculated with *C. scovillei*, L1-7 and L3-5 had very effective control ability on leaves. The results showed that both L1-7 and L3-5 had a controlling effect on disease incidence on pepper leaves.

Discussion

Plant endophytes are a group of microorganisms that are not harmful to host plants and colonize plant tissues, organs or intercellular spaces at a particular stage or throughout their life history(Zhang and Liu 2014). Plant endophytes have been reported to have several biological functions such as secretion of plant hormones, induction of plant resistance, production of antifungal metabolites and nitrogen-fixative (Amareesan 2012). In this study, two strains of antagonistic endophytic bacteria with good antagonism to *C. scovillei* were isolated from the healthy pepper leaves and investigated. They were identified as *B. amyloliquefaciens* and *B. velezensis* in the genus *Bacillus* both of which have nitrogen fixation and IAA secretion functions, and all of which have some salt tolerance. Production of peppers is severely limited by anthracnose disease caused by several *Colletotrichum* species including *C. scovillei* (Damm et al. 2012; Liu et al. 2016a). Therefore, it is imperative to develop biological agents that have a preventive effect on the pathogen. For example, some *Bacillus* strains have been shown to be excellent antagonists against *Colletotrichum*, such as *Bacillus* spp. *B. subtilis*, *B. amyloliquefaciens*, *B. velezensis* (Hassan et al. 2010; Agustín et al. 2018; Prapasri et al. 2018; Reyes-Estebanez et al. 2019). *C. scovillei* was discovered in China only in recent years (Chi et al. 2016), and the studies on endophytic *Bacillus* as a biocontrol strain of *C. scovillei* were not reported. Therefore, the two strains of *B. amyloliquefaciens* and *B. velezensis* investigated in this study were of great importance for control.

Bacillus is one of the dominant endophytic bacteria in plants. Due to its strong ability to inhibit plant pathogenic microorganism, it is often used as a biocontrol agent in agricultural fields(Ruiz-García et al. 2005). *B. amyloliquefaciens* and *B. velezensis* were

important plant growth promoting bacteria in the genus *Bacillus*, which not only have good control effect on plant diseases, but also have a wide range of reports on promoting plant growth and development (Santoyo et al. 2012), such as biological organisms pesticides or biological fertilizers have a wide range of applications (Wei et al. 2018a). Studies had shown that the production of a substance called bacillomycin D by *B. amyloliquefaciens* restricted the growth of mycelium and could not grow normally (Agustín et al. 2018). It was also found that an antifungal protein produced by *Bacillus* sp. induced abnormal hyphal elongation (Prapasri et al. 2018). The research showed that the exocrine antibacterial agent produced by *B. amyloliquefaciens* SSY2 can effectively inhibit the growth of microorganisms (Chen et al. 2018). Other studies also have shown that the n-butanol produced by *B. amyloliquefaciens* HAB-2 deforms and swells the hyphae, thereby effectively inhibiting the pathogen (Wei et al. 2018a). *B. velezensis* was reported by the research worker that it had antagonistic effect on a variety of pathogenic fungi, such as *Arthrinium phaeospermum*, *Fusarium oxysporum*, *Cylindrocladium scoparium*, *Botrytis cinerea* and *Penicillium* (Xu et al. 2014; Sun et al. 2018), and the like. Their conclusions were consistent with the results of our study that *B. amyloliquefaciens* and *B. velezensis* inhibited the growth of *C. scovillei*, indicating that both biocontrol strains L1-7 and L3-5 can produce certain substances that prevent the normal growth of mycelia. *B. amyloliquefaciens* K103 was reported that had high ability to fix nitrogen and solubilize organic phosphorus but the tested strains had no ability to solubilize organic phosphorus (Dong et al. 2018). This difference may be related to the fact that the strains were isolated from different hosts, so the same bacteria have different functions. *B. velezensis* was reported that had the effect of secreting IAA (Cai et al. 2018), which was consistent with the results of our tests. Therefore, IAA promotes plant root development and nutrient uptake to promote plant growth and increase plant resistance. The two *Bacillus* strains isolated in our study all had this function, suggesting that these two antagonistic endophytes can not only prevent and control pathogens, but also develop into high-value growth-promoting bacteria. Soil salinization has become a major problem in global land management (Unger and Kaspar 1994; García and Mendoza 2014; Xun et al. 2015). In arid and semi-arid areas of northern China, saline soils affect crop emergence and restrict crop growth, which affects crop yield and quality (Chinnusamy et al. 2005; Cui et al. 2011). Therefore, it is of great importance for sustainable agriculture in China to develop endophytic control strains that can control diseases well even in saline soils. However, the salt tolerance of *Bacillus* with biological control function has not been reported yet. Therefore, research and development of salt-tolerant biocontrol strains is essential to effectively prevent and control pathogens in this region. In this experiment, both *Bacillus* strains have better salt tolerance, which makes them have stronger ecological adaptability. They can play a greater role in areas with high salinity. In this research, an indoor pot experiment was used to verify the inhibitory effect of two biocontrol strains on *C. scovillei*, and their control effects reached 80.64% and 73.39%, respectively. There was not much difference between the potted plant control test of L1-7 and the plate anti-pathogen test, because the control effect of *C. scovillei* in the pot test was 80.64%, while the plate test showed that the anti-pathogen rate was 79%. But in the pot experiment, the control effect of L3-5 to *C. scovillei* was 73.39%, and the inhibition rate reached more than 80% in the two plate anti-pathogen experiments. The reason for this result may be that in the plate anti-pathogen test, L1-7 effectively controlled its growth in the early stage of the growth of the strain, while L3-5 played a role in the later stage of the growth of the strain. Therefore, endophytic control strains that act in the early stage of pathogen infection have better control effects on plants in pot experiments.

All in all, the antagonistic strains selected in this experiment represent strain resources for the biological control of pepper anthracnose and lay a foundation for better utilization of endophytic bacteria in pepper leaves. However, the types of antibacterial substances and antibacterial mechanisms produced by these two *Bacillus* strains require further investigation.

Declarations

Acknowledgements This study was supported by the project of National Natural Science Foundation of China (No: 31660148) and by the Science and Technology Innovation Fund of Gansu Agricultural University (No. GAU-XKJS-2018-148) from Gansu Agricultural University to Prof. Chengde Yang.

Authors' contributions First of all, I would like to thank my mentor, Professor Yang, for his guidance and support in the experiment. Second, I would like to thank my colleagues Fan, Cui, and Jin for helping me at different stages of my experiment, and Osei for helping me revise the article.

Compliance with ethical standards

Declaration of interest The authors declare no conflict of interest. Each of the authors of the study is aware of and agrees with the submission of this paper.

Ethics declarations The authors solemnly promise that the research meets ethical standards.

References

1. Agustín LB et al (2018) Effects of bacillomycin D homologues produced by *Bacillus amyloliquefaciens* 83 on growth and viability of *Colletotrichum gloeosporioides* at different physiological stages. *Biol Control* 127:145–154
2. Ahmad F, Ahmad I, Khan MS (2008) Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiol Res* 163:173–181
3. Amaresan N (2012) Endophytic bacteria from tomato and chilli, their diversity and antagonistic potential against *Ralstonia solanacearum*. *Archives Of Phytopathology Plant Protection* 45:344–355
4. Asghari S, Harighi B, Mozafari AA, Esmaeel Q, Barka EA (2019) Screening of endophytic bacteria isolated from domesticated and wild growing grapevines as potential biological control agents against crown gall disease. *Biocontrol* 64:723–735
5. Benhamou N, Klopper JW, Quadt-Hallman A, Tuzun S (1996) Induction of Defense-Related Ultrastructural Modifications in Pea Root Tissues Inoculated with Endophytic Bacteria. *Plant physiology* 112:919–929
6. Bhattacharya A et al (2019) Intervention of bio-protective endophyte *Bacillus tequilensis* enhance physiological strength of tomato during *Fusarium* wilt infection. *Biol Control* 139:1–12
7. Bouaoud Y, Troulet C, Foughalia A, Berge O, Aissat K (2018) A multi-criteria approach for the selection of efficient biocontrol agents against *Botrytis cinerea* on tomato in Algeria. *Biocontrol* 63:299–311
8. Cai GL, Zhang F, Ouyang YX, Zhao CS, Peng XH, Jiang AM (2018) Research Progress on *Bacillus velezensis*. *Northern Horticulture*:162–167
9. Chanchaichaovivat A, Panijpan B, Ruenwongsa P (2008) Putative modes of action of *Pichia guilliermondii* strain R13 in controlling chilli anthracnose after harvest. *Biol Control* 47:207–215
10. Chanchaichaovivat A, Ruenwongsa P, Panijpan B (2007) Screening and identification of yeast strains from fruits and vegetables: Potential for biological control of postharvest chilli anthracnose (*Colletotrichum capsici*). *Biol Control* 42:326–335
11. Chen NN, Yin JY, Wang Y (2018) Identification and Physicochemical Properties of Antifungal Substances of *Bacillus amylolyticus* SSY2. *China Animal Husbandry Veterinary Medicine* 45:3221–3228
12. Chi YK, Zhao W, Chen QQ, Swe T (2016) First Report of *Colletotrichum scovillei* Causing Anthracnose Fruit Rot on Pepper in Anhui Province, China. *Plant Disease*:103
13. Chinnusamy V, Jagendorf A, Zhu JK (2005) Understanding and Improving Salt Tolerance in Plants. *Crop Sci* 45:437–448
14. Cui JZ, Cao TT, Zhang JT, Liu QQ, Liu H (2011) Response to salt stresses and assessment of salt tolerability of soybean varieties in emergence and seedling stages. *Acta Ecol Sin* 31:2805–2812
15. Cui LX, Yang CD, Wei LJ, Li TH, Chen XY (2019) Isolation and identification of an endophytic bacteria *Bacillus velezensis* 8 – 4 exhibiting biocontrol activity against potato scab. *Biological Control* 141
16. Cui YZ, Yang XL, Yang CD, Xue L, Zhang JL, Yao YL (2016) Identification and determination of biological functions of endophytic bacteria from alpine pasture against *Phytophthora. infestans*. *Journal of Plant Protection* 43:789–795
17. Damm U, Cannon PF, Woudenberg J, Crous PW (2012) The *Colletotrichum acutatum* species complex. *Stud Mycol* 73:37–113
18. Dilfuza E, Wirth SJ, Shurigin VV, Abeer H, Abd_Allah EF (2017) Endophytic Bacteria Improve Plant Growth, Symbiotic Performance of Chickpea (*Cicer arietinum* L.) and Induce Suppression of Root Rot Caused by *Fusarium solani* under Salt Stress. *Front Microbiol* 8:18–87
19. Ding R, Li MK, Ren GY, Jin YQ, Zhang HQ, Chen XH (2017) Isolation and Identification of Endophytic Fungi From *Liparis japonica* and Screening for Antimicrobial Activities. *Northern Horticulture*:126–130
20. Dong CJ, Wang LL, Li L, Qin YX, Li PL, Shang QM (2018) Growth-Promoting Effects of *Bacillus amyloliquefaciens* K103 on Cucumber Plug Seedlings. *Acta Horticulturae Sinica* 45:2188–2198

21. Dong XZ, Cai MY (2001) Manual of System Identification of Common Bacteria. China Science Press, Beijing
22. El-Deeb B, Fa Yez K, Gherbawy Y (2013) Isolation and characterization of endophytic bacteria from medicinal plant in Saudi Arabia desert and their antimicrobial activities. *J Plant Interact* 8:56–64
23. Fang ZD (1998) Methods of Research on Plant Diseases, 3 edn. agricultural publishing house, Beijing
24. García I, Mendoza R (2014) Lotus tenuis seedlings subjected to drought or waterlogging in a saline sodic soil. *Environ Exp Bot* 98:47–55
25. Glickmann E, Dessaux Y (1995) A critical examination of the specificity of the salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Applied Environmental Microbiology* 61:793–796
26. Gravel V, Martinez C, Antoun H, Tweddell RJ (2005) Antagonist Microorganisms with the Ability to Control *Pythium* Damping-off of Tomato Seeds in Rockwool. *Biocontrol* 50:771–786
27. Hallmann J, Mahaffee WF, Kloepper JW, Quadthallmann A (1997) Bacterial endophytes in agricultural crops. *Can J Microbiol* 43:895–914
28. Hao XJ (2010) endophyte. China Agricultural Science and Technology Press, Beijing
29. Hassan ED (2017) Plant growth-promoting activities for bacterial and fungal endophytes isolated from medicinal plant of *Teucrium polium* L. *J Adv Res* 8:687–695
30. Hassan MN, Afghan S, Hafeez FY (2010) Suppression of red rot caused by *Colletotrichum falcatum* on sugarcane plants using plant growth-promoting rhizobacteria. *Biocontrol* 55:531–542
31. Hirooka T, Ishii H (2013) Chemical control of plant diseases. *J Gen Plant Pathol* 79:390–401
32. Jiang GF, Song L (2014) Advances on Biologically Controlling Pepper Anthracnose. *Hubei Agricultural Sciences* 53:2481–2484
33. Khl J, Kolnaar R, Ravensberg WJ (2019) Mode of Action of Microbial Biological Control Agents Against Plant Diseases: Relevance Beyond Efficacy. *Front Plant Sci* 10:845
34. Liotti RG, Figueiredo M, Soares MA (2019) *Streptomyces griseocarneus* R132 controls phytopathogens and promotes growth of pepper (*Capsicum annum*). *Biol Control* 138:104065
35. Liu FL et al (2016a) Molecular and phenotypic characterization of *Colletotrichum* species associated with anthracnose disease in peppers from Sichuan Province, China. *Scientific reports* 6:732–761
36. Liu HY, Du FY, Xiao L, Jin YL (2016b) Isolation and Antifungal Activity of Endophytic Fungi Derived From *Rauvolfia verticillata*. *Northern Horticulture*:105–107
37. Liu YH et al (2016c) Culturable endophytic bacteria associated with medicinal plant *Ferula songorica*: molecular phylogeny, distribution and screening for industrially important traits. *Biotech* 6:209
38. Mnasri N et al (2016) Efficacy of some rhizospheric and endophytic bacteria in vitro and as seed coating for the control of *Fusarium culmorum* infecting durum wheat in Tunisia. *Eur J Plant Pathol* 147:501–515
39. Morris CE et al (2008) The life history of the plant pathogen *Pseudomonas syringae* is linked to the water cycle. *Isme Journal* 2:321
40. Peters LP, Prado LS, Silva F, Souza F, Carvalho CM (2020) Selection of endophytes as antagonists of *Colletotrichum gloeosporioides* in aai palm. *Biol Control* 150:104350
41. Prapasri S, Kongyuth L, Rapeewan S, Panan R (2018) *Bacillus* sp. strain M10 as a potential biocontrol agent protecting chili pepper and tomato fruits from anthracnose disease caused by *Colletotrichum capsici*. *Biocontrol* 63:833–842
42. Prisana et al (2019) *Streptomyces angustmyceticus* NR8-2 as a potential microorganism for the biological control of leaf spots of *Brassica rapa* subsp. *pekinensis* caused by *Colletotrichum* sp. and *Curvularia lunata*. *Biological Control* 138:104046–104046
43. Rai C et al (2009) *Colletotrichum* – names in current use. *Fungal Divers* 39:147–182
44. Reyes-Estebanez MM, Sanmartín P, Camacho-Chab JC, Rosa-García S, Morales OO (2019) Characterization of a native *Bacillus velezensis*-like strain for the potential biocontrol of tropical fruit pathogens. *Biological Control* 141
45. Ruiz-García C, Béjar V, Martínez-Checa F, Llamas I, Quesada E (2005) *Bacillus velezensis* sp. nov., a surfactant-producing bacterium isolated from the river Vélez in Málaga, southern Spain. *Int J Syst Evol Microbiol* 55:191–195

46. Santoyo G, Orozco-Mosqueda MDC, Govindappa M (2012) Mechanisms of biocontrol and plant growth-promoting activity in soil bacterial species of *Bacillus* and *Pseudomonas*: a review. *Biocontrol Sci Tech* 22:855–872
47. Silva D, Groenewald JZ, Crous PW, Ades PK, Taylor P (2019) Identification, prevalence and pathogenicity of *Colletotrichum* species causing anthracnose of *Capsicum annuum* in Asia. *IMA Fungus* 10:2–32
48. Sun PP, Cui JC, Jia XH, Wang WH (2018) Complete genome analysis of *Bacillus velezensis* L-1 and its inhibitory effect on pear gray and blue mold. *Acta Microbiologica Sinica* 58:1637–1646
49. Unger PW, Kaspar TC (1994) Soil Compaction and Root Growth: A Review. *Agron J* 86:759–766
50. Vetrivelkai P, Sivakumar M, Jonathan E (2010) Biocontrol potential of endophytic bacteria on *Meloidogyne incognita* and its effect on plant growth in bhendi. *Journal of Biopesticides* 3:452–457
51. Wei DD, Miao WG, Sun QQ, Wu GL, Liu WB, Jin PF (2018a) Analysis of the active components of *Bacillus amyloliquefaciens* HAB-2 against *Colletotrichum gloeosporioides*. *Journal of Fruit Science* 35:1253–1261
52. Wei LJ, Wang YQ, Yang CD, Xue L (2018b) Biological Function Identification and Determination of Endophytic Bacteria in Leaves of *Kobresia capillifolia* in Haiyan County, Qinghai Province. *Acta Agrestia Sinica* 26:764–769
53. Wheeler BEJ, Kent GC (1969) An Introduction To Plant Diseases. *Quarterly Review of Biology*
54. Xu T, Zhu TH, Li SJ, Qiao TM (2014) Fungus-inhibitory Activity and Gene Cloning of β -glucanase from *Bacillus velezensis* YB15. *Chinese Journal of Biological Control* 30:276–281
55. Xun F, Xie B, Liu S, Guo C (2015) Effect of plant growth-promoting bacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) inoculation on oats in saline-alkali soil contaminated by petroleum to enhance phytoremediation. *Environmental Science Pollution Research* 22:598–608
56. Zhang XJ, Liu RJ (2014) Broad and narrow definition of endophytes and related advances in the study. *Microbiology China* 41:560–571
57. Zhao ZL, Dorje G, Wang ZT (2010) Identification of medicinal plants used as Tibetan Traditional Medicine Jie-Ji. *J Ethnopharmacol* 132:122–126

Tables

Table 1
Classification standard of pepper anthracnose disease

Disease coefficient	Representative value	Classification standard
0	0	No signs of disease
1	1	Only the tender shoots show signs of disease
2	2	Less than 25% of leaves, leaf buds, flower buds or fruits are susceptible to disease
3	3	25%-30% of leaves, leaf buds, flower buds or fruits are susceptible to disease
4	4	More than 50% of leaves, leaf buds, flower buds or fruits are susceptible to disease

Table 2
Biological functions determination of antagonistic endophytic bacteria L1-7 and L3-5

Strain	Inhibition rate(%)	Nitrogen fixation	Phosphorus solubilizing		Secreting IAA($\mu\text{g mL}^{-1}$)								
			Inorganic phosphorus	Organic phosphorus	Qualitative determination				Quantitative determination				
					With tryptophan	Without tryptophan	With tryptophan	Without tryptophan					
PC	S2	PC	S2	PC	S2	PC	S2	PC	S2	PC	S2	PC	S2
L1-7	79	+	□	□	+	+	+	+	0.054	1.048	0.055	1.048	
L3-5	80	+	□	□	+	+	+	+	0.054	1.049	0.056	1.047	

Note: "+": With biological function, "-": Without biological function

Table 3
Effects of different endophytic bacterial on disease control of *C. scovillei* in pot experiment

Treatment	*	
	Disease Index	Control efficiency(%)
CK	86.11 \pm 3.67a	—
L1-7	16.67 \pm 4.17b	80.64 \pm 4.84a
L3-5	22.92 \pm 2.08b	73.39 \pm 2.42a

*: The results are presented as mean of three independent experiments \pm standard error. Values of each column followed by a different letter indicate significant differences ($P < 0.05$) according to LSD test. Values with shared letter are not significantly different ($P < 0.05$)

Figures

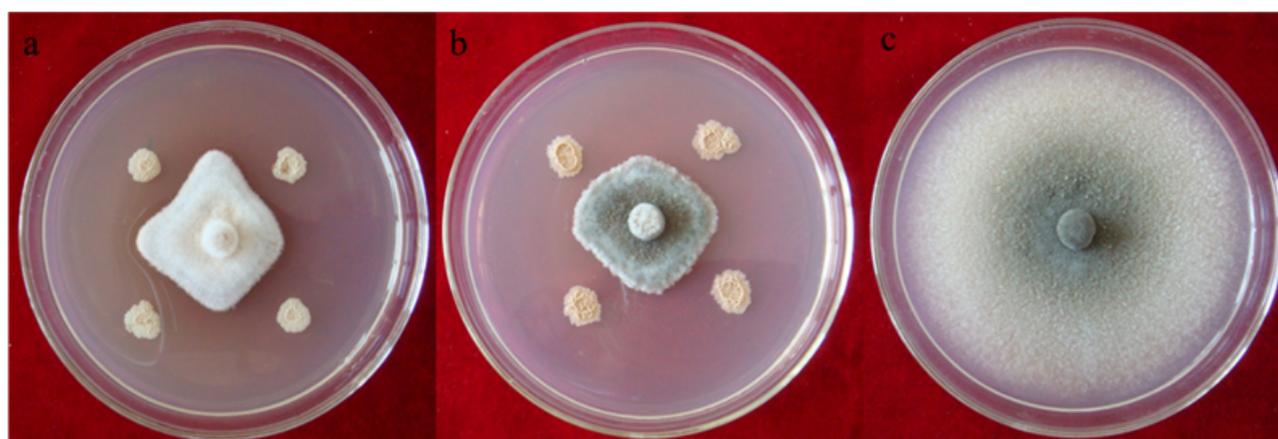


Figure 1

Inhibition of *C. scovillei* by antagonistic strains L1-7 and L3-5. a: The inhibitory effect of L1-7 on *Colletotrichum scovillei*; b: The inhibitory effect of L1-7 on *Colletotrichum scovillei*; c: The control

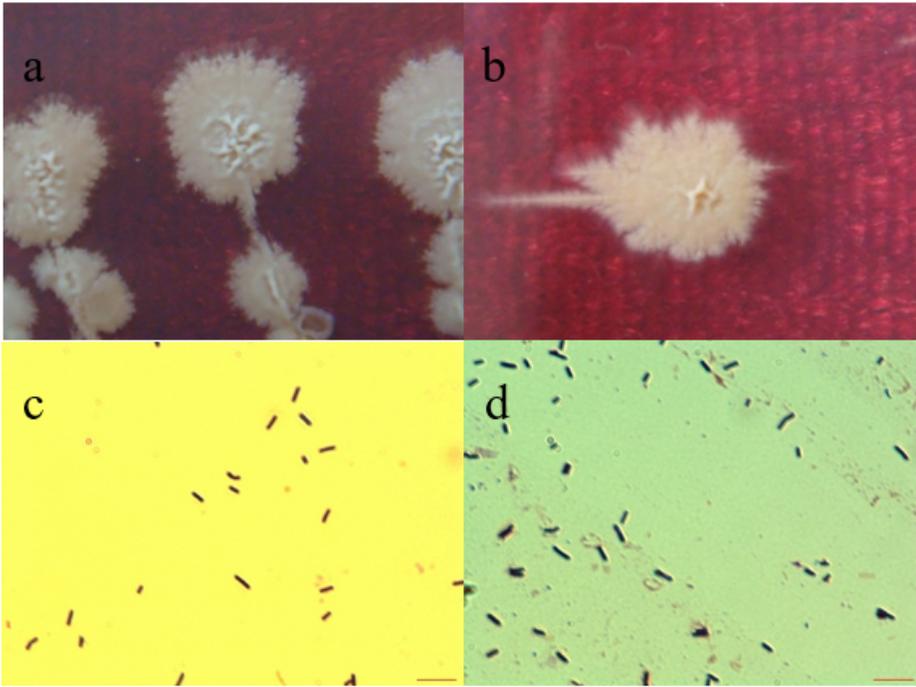


Figure 2

Culture traits and gram-stained characteristics of antagonistic strains L1-7 and L3-5. a: Culture traits of L1-7; b: Culture traits of L3-5; c: Gram staining of strain L1-7(100×); d: Gram staining of strain L3-5(100×). Scale bars = 4 μm for (c and d)

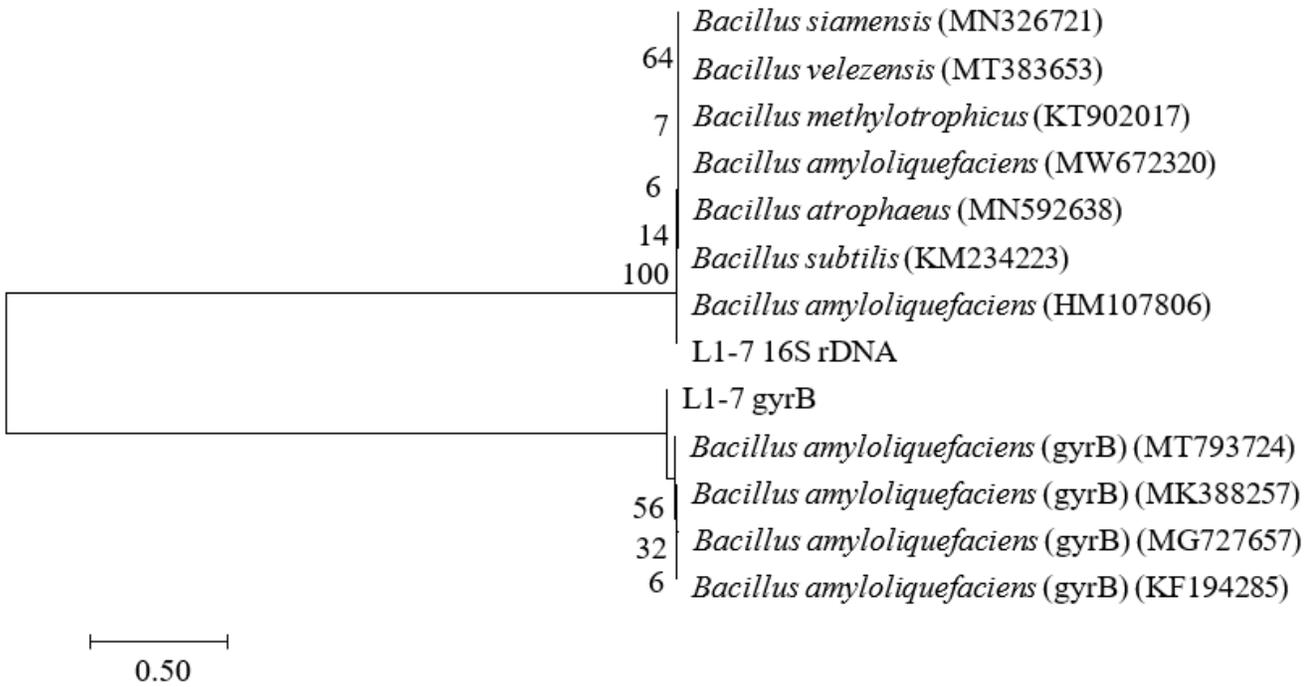


Figure 3

Phylogram generated from Neighbor-Joining analysis based on alignment of partial sequences of 16S rDNA and gyrB gene, showing the phylogenetic relationships of *Bacillus* species to the isolate L1-7. Among them, 'L1-7 16S rDNA' was the sequence of amplification by the universal primer. 'L1-7 gyrB' was the sequence of amplification by the gyrB primer

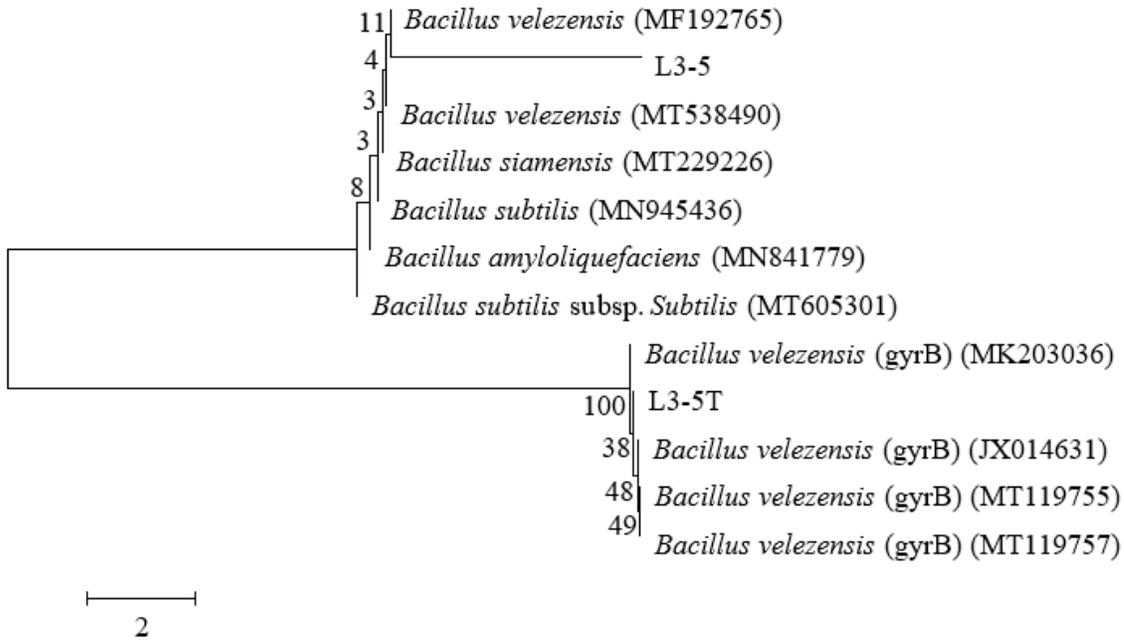


Figure 4

Phylogram generated from Neighbor-Joining analysis based on alignment of partial sequences of 16 S rDNA and gyrB gene, showing the phylogenetic relationships of *Bacillus* species to the isolate L3-5. Among them, 'L3-5 16S rDNA' was the sequence of amplification by the universal primer. 'L3-5 gyrB' was the sequence of amplification by the gyrB primer

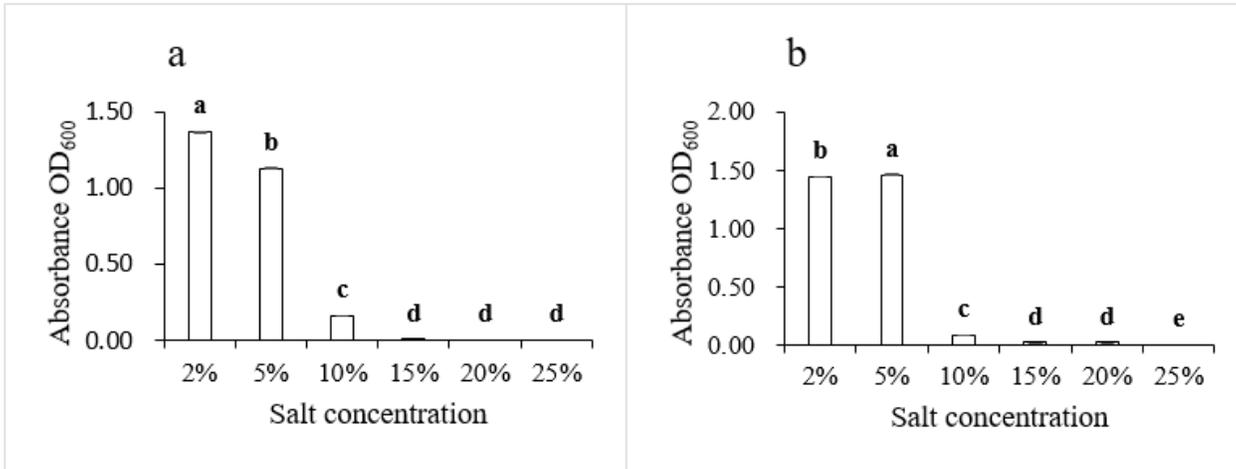


Figure 5

Salt tolerance of antagonistic strains L1-7 and L3-5. a: Salt tolerance of strain L1-7; b: Salt tolerance of strain L3-5. Different lower case letters above bars indicate significant differences ($P < 0.05$) among according to LSD test and Values with shared letter are not significantly different ($P < 0.05$)



Figure 6

Results of pot experiment. a: After inoculation with pathogenic fungi, the leaves treated with fermentation liquid L1-7 was sprayed; b: After inoculation with pathogenic fungi, the leaves treated with fermentation liquid L3-5 was sprayed; c: Leaves untreated after inoculation with pathogenic fungi; d: A blank control without any treatment