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Antifungal Activity of Rhizosphere Bacillus Isolated from *Ziziphus jujuba* against *Alternaria alternata*

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Abstract The serious impact of *Alternaria alternata* on jujube black spot disease has seriously affected the quality and yield of jujube, constraining the sustainable development of the jujube industry. The purpose of this study was to isolate and screen highly effective biocontrol strains of jujube black spot disease from jujube rhizosphere soil, enriching the biocontrol bacterial resources of jujube black spot disease, and laying the foundation for the development of biocontrol agents for jujube black spot disease. Thirty-three soil samples were collected from 4 regions in southern Xinjiang using the five-point sampling method. The strains with antagonistic effects were isolated and screened by dilution spread method and plate confrontation method, and identified by morphological, physiological, biochemical characteristics, as well as 16S rDNA, gyrB, and rpoB gene sequences. Indoor and field efficacy experiments were conducted to determine their biocontrol effect. A total of 110 strains with antibacterial activity were selected, and one strain *Bacillus velezensis* 26-8 with stable antagonistic effect was further tested. Biological characteristic experiments showed that strain 26-8 could grow at NaCl concentrations of 0.5%-10% and pH 4.0-9.0. The biocontrol experiment results showed that *Bacillus velezensis* 26-8 could achieve an 89.83% control effect against black spot disease. In conclusion, strain 26-8 has good salt and alkali tolerance, exerts a good control effect on jujube black spot disease, and is worthy of further study.

Keywords: jujube fruit black spot disease; *Bacillus velezensis*; growth characteristics; control effect

1. Introduction

The jujube (*Ziziphus jujube* Mill) is a natural medicinal and edible plant, rich in various nutrients such as vitamins, amino acids, proteins [1,2]. The jujube industry is one of the key pillar industries for the economic development in Xinjiang, China. However, in recent years, the appearance of jujube black spot disease has hindered the industry's development, causing significant economic losses to fruit farmers, and seriously affecting the quality and yield of jujube fruits [3]. Currently, chemical control has become the main method for plant disease prevention and control, but long-term use of chemical pesticides can pollute the environment and pose a threat to human health. Therefore, biocontrol as a new green and non-polluting control method is receiving increasing attention and is expected to gradually replace chemical control [3–5].

The spore-forming *Bacillus* is the most widely used biocontrol agent currently due to its fast reproduction and strong stress resistance. As a branch of *Bacillus*, *Bacillus velezensis* has been extensively studied and shown to have good disease prevention and growth-promoting effects [6]. For example, Zhu Li et al. found that *Bacillus velezensis* SM905 powder had an 80.74% prevention effect on iron tuber charcoal rot, which was better than the effect of carbendazim [7]. Zhang Nuoni et

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al. discovered that *Bacillus velezensis* ZF-10 had a relative prevention effect of 61.85% on tobacco potato Y virus in pot trials [8]. Li et al. found that *Bacillus velezensis* Ba-0321 had a prevention effect of 81.00% on tobacco root rot [9]. These studies indicate that *Bacillus velezensis* is a strain with potential for biocontrol against various diseases, and it has good application prospects. Currently, there are limited reports on the control of jujube black spot disease by *Bacillus velezensis* in the field, and there are relatively few products that are stable in efficacy. The actual application is influenced by many external factors such as soil environment, geographical climate [10–13]. Further selection of strains with better adaptability is of great significance for expanding biocontrol agent resources.

This study used the rhizosphere soil of Xinjiang jujube as the experimental material, isolated and screened *Bacillus* strains with antibacterial activity against *Rhizoctonia solani*, identified the strains by morphological, physiological, biochemical characteristics, and molecular biology methods, clarified their growth characteristics, and studied the in vitro control effect of the strain on jujube black spot disease, providing excellent germplasm resources for the biological control of jujube black spot disease.

2. Materials and Methods

2.1. Isolation of Soil Microorganisms

Five-point sampling method was used to collect rhizosphere soil samples from jujube orchards in Shaya, Wensu, Moyu and Zepu of Xinjiang. Weigh 10.0g soil sample, transfer to a triangular flask containing 90mL sterile water and appropriate amount of glass beads, shake at a speed of 150r/min for 20min on a shaker to prepare soil suspension, dilute the soil suspension using a 10-fold dilution method to prepare concentrations of 10^{-3}, 10^{-4}, and 10^{-5}, then place them in a constant temperature water bath at 80℃ for 15min, evenly spread 200μL of the soil dilution on NA agar plates, let stand for 20min, then invert the plates and incubate at 30℃ for 48h in a constant temperature incubator, with 3 replicates for each treatment. Select representative single colonies for purification and cultivation.

2.2. Antimicrobial Screening

Using the flat confrontation method to screen for antagonistic *Bacillus* spp. [14]. *Alternaria alternata* was isolated and preserved from the junction of diseased and healthy jujube fruits by this experiment with the pathogen of jujube black spot as the target bacteria. Use a sterile hole punch to take 6 mm agar discs of *A. alternata*, place them in the center of the PDA plate, spot the antagonistic bacteria at four corners 2 cm away from the center, with 3 parallel strains per strain, and incubate at a constant temperature of 28°C for 5 days. Observe whether each strain has antibacterial effect, and record the strains with antibacterial activity.

Refer to the method of Yang Di et al. [15] for the secondary screening of the initial strains. Inoculate 100μL of *A. tenuissima* spore suspension on PDA medium and let stand for 10 minutes. Using a sterile hole puncher, make 4 equidistant holes 1.5cm from the center of the PDA plate using the cross method, and add 100μL of each antagonistic bacterial sterile filtrate to each hole, and culture at 28℃ for 5 days. Three replicates per strain. Observe the presence of inhibition zones and measure the diameter of the inhibition zone using the cross method.

2.3. Strain Identification

The morphological, physiological and biochemical identification methods of antagonistic bacteria are as follows. Inoculate the purified antagonistic bacteria into NA medium and cultivate at 30℃ for 2 days. Observe the morphological characteristics of the colonies. Pick the colonies for Gram staining to observe the morphological characteristics of the bacterial cells. The physiological and biochemical tests were conducted according to the methods [16–19].

The molecular biological identification methods of antagonistic bacteria are as follows. Use the DNA extraction kit for bacterial genome DNA to extract antagonistic bacteria genome DNA. Use the bacterial genome DNA as a template, amplify the target fragments with universal primers for bacteria

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16S rDNA, *gyr*B, and *rpo*B, detect the PCR amplification products by 1.7% agarose gel electrophoresis, observe the clarity of the electrophoresis bands using a gel imaging system [20,21]. For clear amplification product bands, send them to Shanghai Sangon Biotech Co., Ltd. for sequencing. Use Seq Man software to assemble the sequences, then submit them to the NCBI database for similarity comparison analysis with BLAST software. Construct a phylogenetic tree using the Neighbor-Joining method in MEGA7.0 software. Determine the classification status of the strains. The amplification system and conditions are shown in Table 1.

Table 1. PCR amplification system and conditions.

Gene segment	Primer	Reaction system	Reaction conditions
16S rDNA	27F(5'-AGAGTTTGATCCTGGCTCAG-3') 1492R(5'-GGTTACCTTGTTACGACTT-3')		95℃pre-denaturation 5min。95℃degeneration 30s ,58℃annealing 15s ,72℃elongation 2min,20cycles。72℃ elongation 10min。
gyrB	UP-1(5'-GAAGTCATCATGACCGTTCTG CAYGCNGGNGGNAARTTYGA-3') UP-2r(5'-AGCAGGGTACGGATGTGCGAG CCRTCNACRTCNGCRTCNGTC AT-3')	Mix 12.5 μL primer 0.5 μL template 1.0 μL ddH_2O 11.0 μL	95℃pre-denaturation 5min。94℃degeneration 1min,54℃annealing 1min,72℃elongation 2min,25cycles。72℃ elongation 10min。
rpoB	f(5'-AGGTCAACTAGTTCAGTATGG AC-3') r(5'-AAGAACCGTAACCGGCAACTT -3')		94℃pre-denaturation 4min。94℃degeneration 1min,51℃annealing 1min,72℃elongation 1min,25cycles。72℃ elongation 10min。

2.4. Determination of Antagonistic Bacterial Strain Growth Characteristics.

The determination method of antagonistic bacteria growth curve is as follows. Inoculate the antagonistic bacteria activated into 50mL NB medium, shake culture at 28°C, 180r/min for 24h to obtain seed solution. Inoculate the seed solution with 1% inoculum size into NB medium shake culture at 28°C, 180r/min, sampling every 2h to measure OD600nm value using a spectrophotometer, with sterile NB medium as control, 3 replicates for each treatment.

The determination method of the effect of temperature on the growth of antagonistic bacteria is as follows. Inoculate the antagonistic bacteria into NB medium at a rate of 1%, and shake culture at 24℃, 28℃, 32℃, 36℃, and 40℃ under 180r/min for 24 hours. The viable bacterial count was determined by plate counting.

The method for measuring the effect of pH on the growth of antagonistic bacteria is as follows. Prepare NB culture medium with pH of 4, 5, 6, 7, 8. Incubate at 28°C and 180r/min for 24 hours. Determine the number of viable bacteria using the plate counting method.

The determination method of the effect of NaCl concentration on the growth of antagonistic bacteria is as follows. Prepare NB culture medium with NaCl concentrations of 0.5%, 1%, 2%, 5%, 7%, and 10%, and culture at 28℃ and 180r/min for 24 hours. Determine the number of viable bacteria using the plate count method.

2.5. Evaluation of Strain Biocontrol Ability

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The determination method of the effect of strain 26-8 on the spore germination of *A. alternata* was as follows. Adjust the concentration of the *A. alternata* spore suspension to 1×10^6/mL with sterile water. The experiment was divided into the following treatments: control: 1mL of pathogenic spore suspension and 1mL of NB culture medium; treatment group: pathogenic spore suspension mixed with the supernatant of the antagonistic bacteria fermentation at a ratio of 1:1. Each treatment was cultured at 28°C for 1 day, 30μL of the mixture was taken for observation under a microscope, a total of 500 spores were observed to calculate the spore germination rate, with 3 replicates for each treatment. Spore germination inhibition rate (%) = (Control spore germination rate - Treatment spore germination rate) / Control spore germination rate × 100.

The determination method of the effect of strain 26-8 on the mycelial growth of *A. alternata* is as follows. Inoculate 100μL of antagonistic bacteria sterile filtrate on PDA plates, then place a 6mm diameter fungus plugs in the center of the PDA plates. Use sterile filtrate without antagonistic bacteria as the control. Cultivate at 28℃ for 5 days, measure the diameter of the pathogenic bacteria colony, and calculate the mycelial growth inhibition rate. Three replicates for each treatment. Mycelial growth inhibition rate (%) = (control colony diameter - treatment colony diameter) / control colony diameter × 100.

The experimental methods of jujube fruit disease prevention in vitro are as follows. Wash the jujube fruits with sterile water, then soak them in 2% sodium hypochlorite solution for 5 minutes, and finally rinse them 3 times with sterile water and air dry naturally. Make a deep wound of about 3 mm near the equator of the jujube fruit using a sterile needle. Inoculate with 10μL of *A. alternata* spore suspension, culture at 25°C for 24h, and then inoculate with 10μL of antagonist solution. Use NB culture medium as a control after inoculation, place in a plastic box, seal with polyethylene film, and culture at 25°C with a relative humidity of 60%. Repeat the process 3 times, each time with 10 jujube fruits, and observe the diameter of jujube fruit lesions every 3 days. Measure lesion diameter using a cross method and calculate the average. Inhibition rate(%) = [(Control lesion diameter - Treated lesion diameter) / Control lesion diameter] × 100.

2.6. Statistical analysis

Data were subjected to standard analysis of variance (ANOVA) and Duncan multiple comparison tests using SPSS software. When $P<0.05$, the difference was considered significant.

3. Results

3.1. Isolation and Screening of Strains

By using the dilution spread method to isolate soil samples from 4 regions in Xinjiang, different morphological single colonies were picked for purification. The strains were preliminarily screened by plate confrontation method, and 133 strains with antibacterial effects were initially selected. By using the inhibition zone method for rescreening, 110 strains with antibacterial activity were selected, and the antagonistic bacteria were classified into 5 categories according to the size of the inhibition zone diameter. There were 14 strains with inhibition zone diameters of 0-5 mm, 10 strains with 5-10 mm, 14 strains with 10-15 mm, 28 strains with 15-20 mm, and 44 strains with 20-30 mm (Table 2). Strain 26-8 with relatively stable antibacterial activity was selected, and its inhibition zone diameter was 25.37±0.37 mm (Figure 1).

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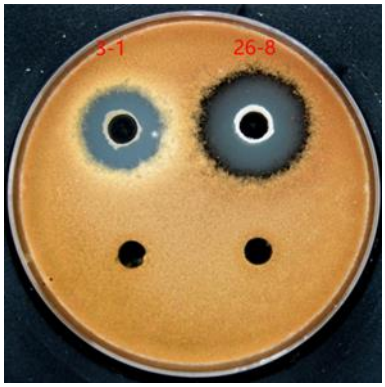

Figure 1. Inhibitory effect of antagonistic bacteria on Alternaria.

Table 2. Inhibitory activity of 110 antagonistic strains against Alternaria.

Antifungal activity	Inhibition diameter (mm)	Number of strains
-	0-5	14
+	5-10	10
++	10-15	14
+++	15-20	28
++++	20-30	44

Note: ++++: Very strong; +++: Strong; ++: Middle; +: Weak; -: Very weak.

3.2. Identification of Antagonistic Strains

Strain 26-8 forms circular or irregular colonies on NA agar plate, with a rough white opaque surface, rod-shaped Gram-positive bacteria (Figure 2). Physiological and biochemical tests revealed that strain 26-8 showed positive results in methyl red test, V-P test, catalase test, oxidase test, nitrate reduction test, and H_2S gas production test. Strain 26-8 could hydrolyze starch, liquefy gelatin, ferment glucose, utilize citrate, D-mannose, D-mannitol, D-xylose, but not utilize L-arabinose (Table 3). Based on its morphological and physiological characteristics, combined with the common bacterial system identification manual, strain 26-8 was preliminarily identified as a *Bacillus* sp.

Through the analysis of the 16S rDNA, *gyr*B, and *rpo*B gene sequences of strain 26-8, segments of approximately 1500bp, 1200bp, and 400-600bp can be amplified, respectively (Figure 3). The sequencing results were submitted to the NCBI database for BLAST comparison analysis, and gene accession numbers were applied for. The Neighbor-Joining method in MEGA7.0 software was used to construct a phylogenetic tree (Figure 4). The results showed that all three gene sequences clustered together with *Bacillus velezensis*, with similarities of 99.28%, 99.64%, and 100% respectively. Combined with morphological and physiological biochemical characteristics, strain 26-8 was identified as *Bacillus velezensis*.

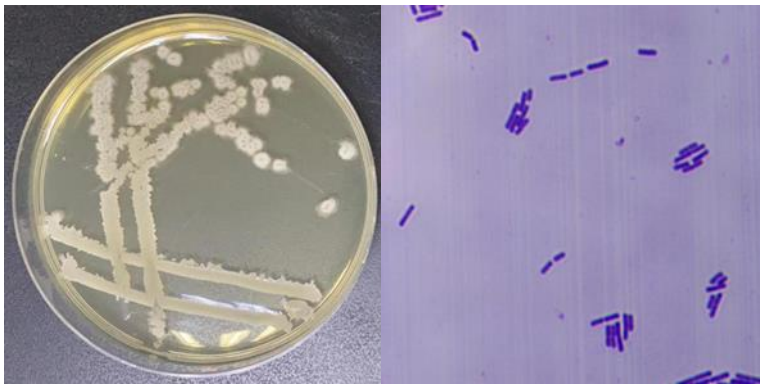

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Figure 2. Colony morphology(A) and Gram staining (Under 10 × 100 times optical microscope) (B) of strain 26-8. Bar = 5 μm.

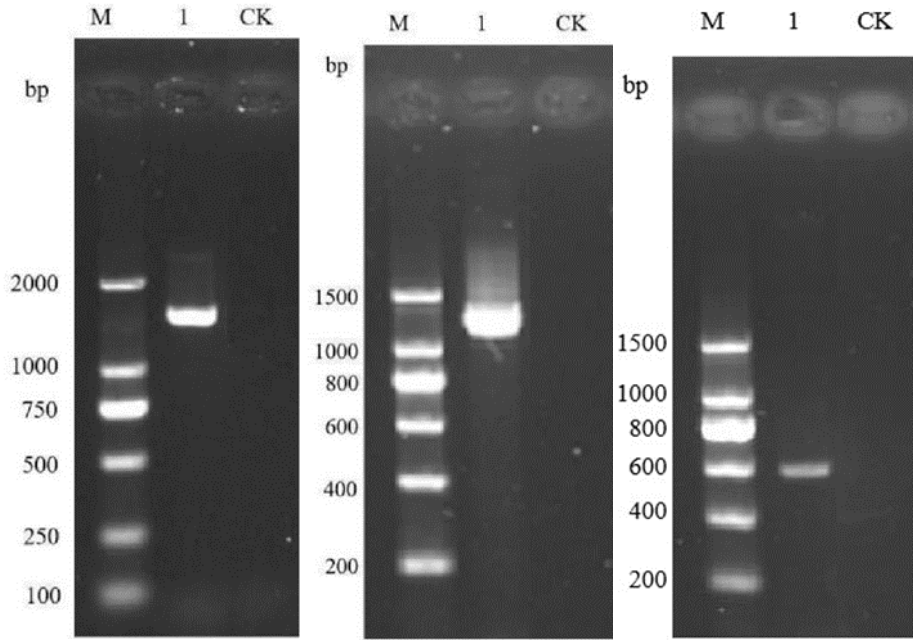
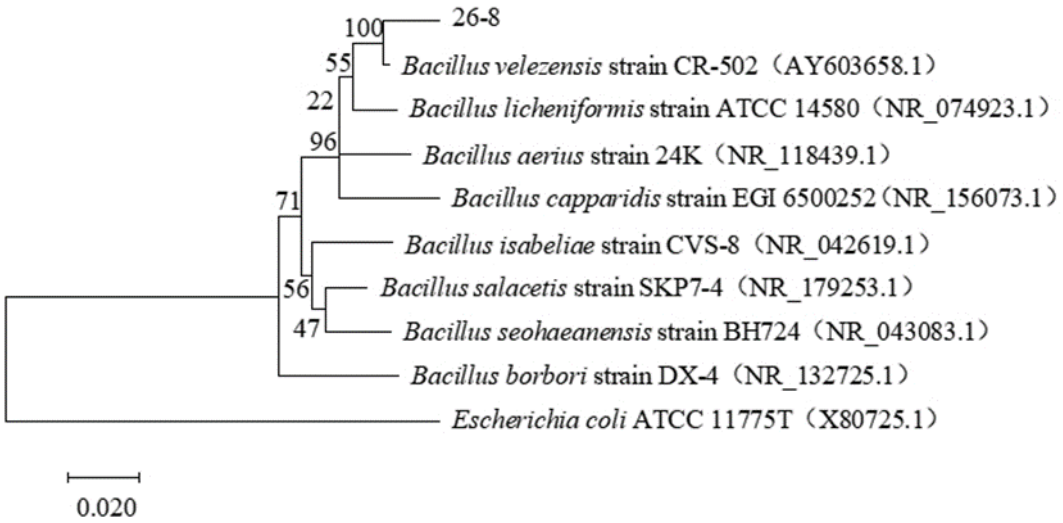


Figure 3. PCR gel electrophoresis based on 16S rDNA(A)、gyrB(B)、rpoB(C) gene sequence.



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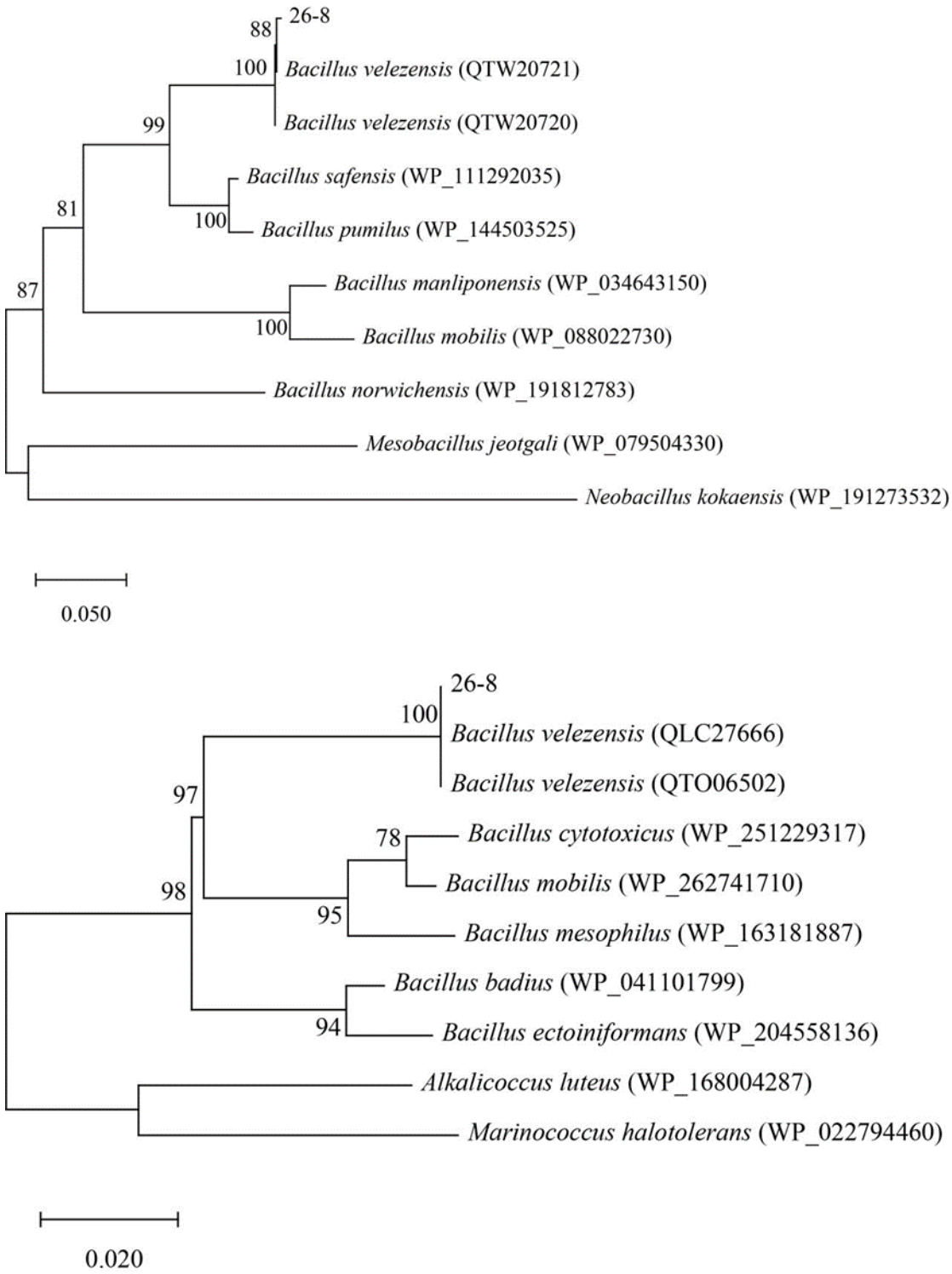

Figure 4. Phylogenetic tree based on 16S rDNA(A)、gyrB(B)、rpoB(C) gene sequence. Branch termini are tagged in light of isolate species and GenBank accession numbers. The numbers above (or below) the nodes show the bootstrap values (50%) occurring after 1000 replications. Scale bars represent the average number of nucleotide substitutions per site.

Table 3. Physiological and biochemical characteristics of strain 26-8.

Items	26-8
Methyl red test	+
V-P text	+
Catalase test	+

 doi:10.20944/preprints202410.0957.v1

Oxidse test	+
Amylolysis	+
Nitrate reduction	+
H_2S production	+
Citrate solution test	+
Gelatin liquefaction	+
D-mannose	+
D-mannitol	+
D-xylose	+
D-arabinose	-
Oxidative fermentation of glucose	fermentation

Note：+: Positive; −: Negative.

3.3. Determination of Strain Growth Characteristics

From Figure 5, it can be seen that from 0-4h, *Bacillus velezensis* 26-8 grows slowly, from 4-16h the strain 26-8 grows rapidly, entering the logarithmic growth phase. After 18h, the biomass of strain 26-8 tends to stabilize, with OD600nm values of 1.7812. According to Figure 6, strain 26-8 can grow in the range of 24℃-40℃. The maximum viable count of strain 26-8 is 7.6×10^8 CFU/mL at 28℃. According to Figure 7, *B. velezensis* 26-8 can grow at pH 4.0-9.0. The highest viable count of strain 26-8 is 8.89×10^8 CFU/mL at pH 7. Growth of the strain is inhibited at pH below 5.0 or above 9.0. As shown in Figure 8, the strain 26-8 can grow well in NaCl concentrations ranging from 0.5% to 5%, with no significant difference in growth observed. The viable cell count is lower at 7% and 10% salt concentrations, but the strain can still grow slowly under high salt conditions, with viable cell counts of 3.0×10^8 CFU/mL and 6.23×10^4 CFU/mL, respectively.

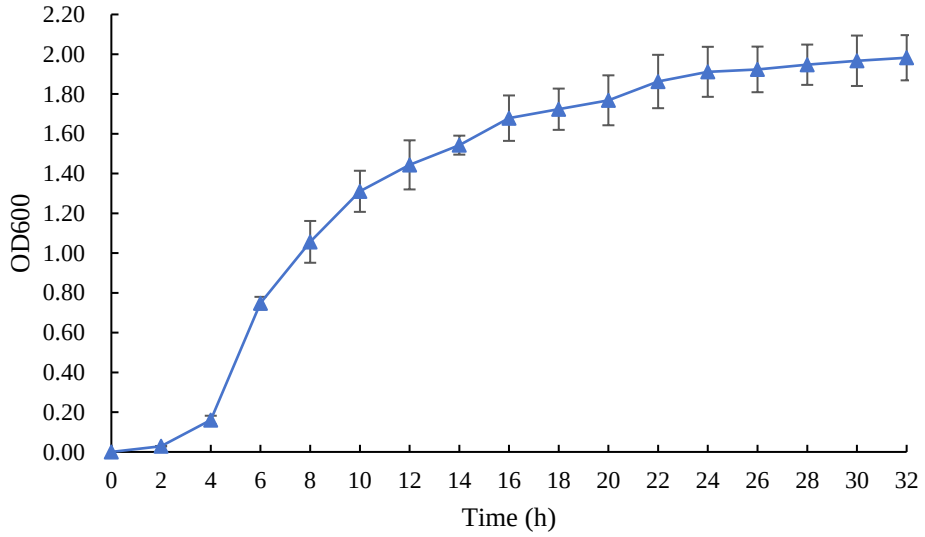

Figure 5. Growth curve of strain 26-8.

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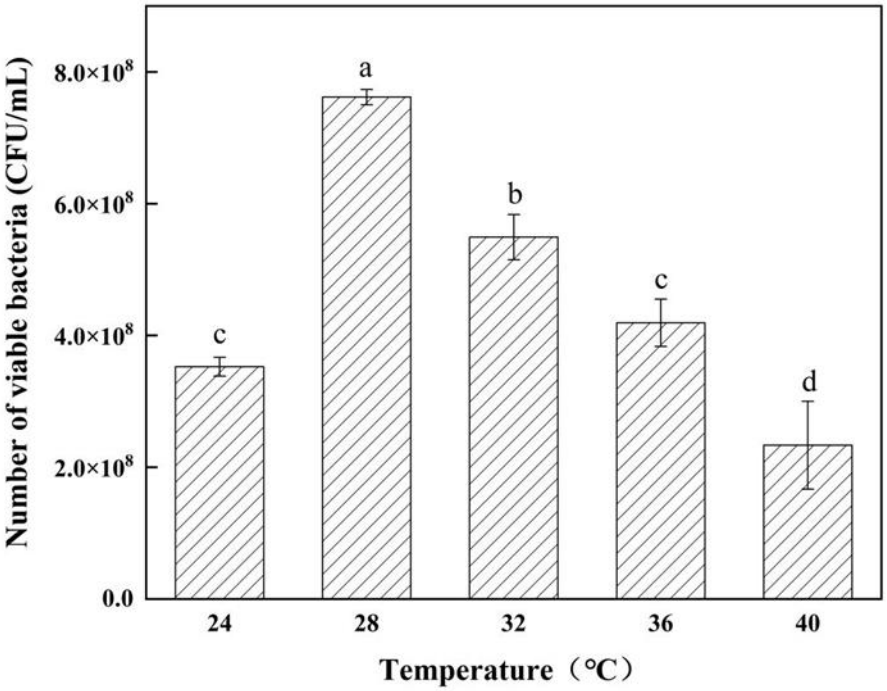

Figure 6. Effect of temperature on the growth of strain 26-8. Different lowercase letters represent significant differences between treatments（p<0.05）. Data marked with the same letter on the columns indicate no significant difference (P≥0.05) according to Duncan's multiple comparison tests.

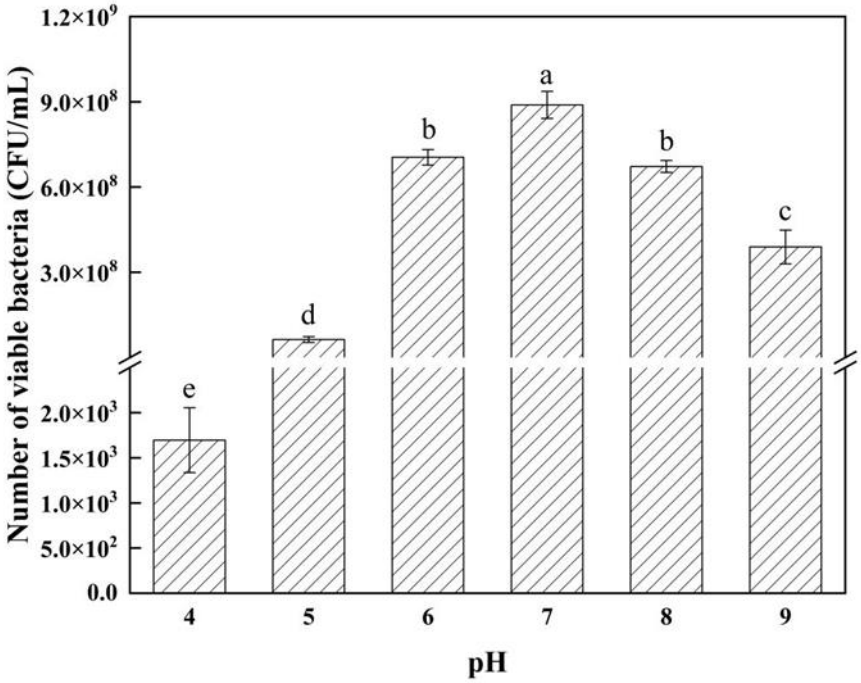

Figure 7. Effect of pH on the growth of strain 26-8. Different lowercase letters represent significant differences between treatments（p<0.05）. Data marked with the same letter on the columns indicate no significant difference (P≥0.05) according to Duncan's multiple comparison tests.

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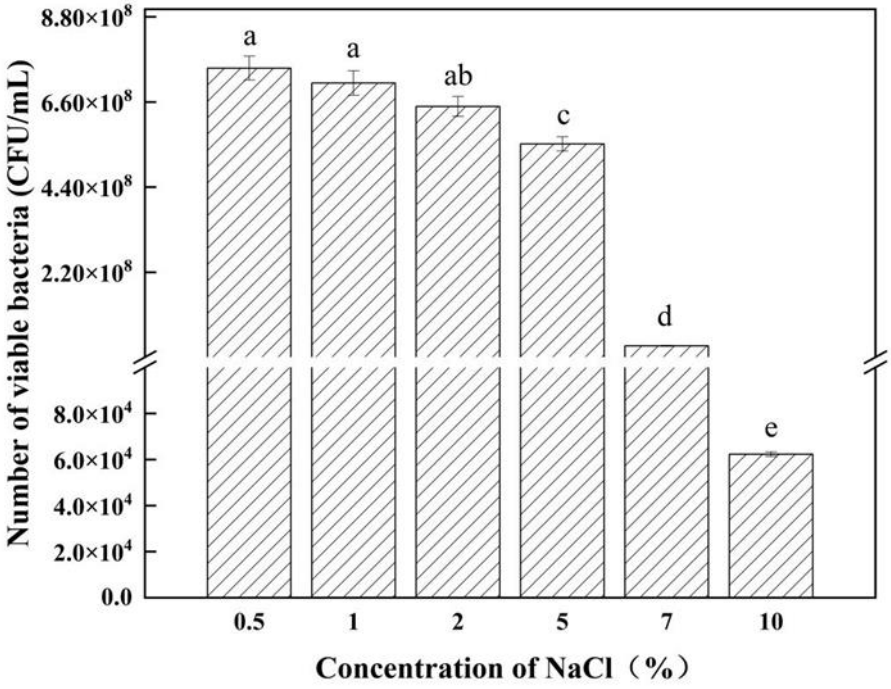

Figure 8. Effect of NaCl concentration on the growth of strain 26-8. Different lowercase letters represent significant differences between treatments（$p<0.05$）. Data marked with the same letter on the columns indicate no significant difference ($P\geq0.05$) according to Duncan's multiple comparison tests.

3.4. Effects of Strains on Spore Germination and Mycelial Growth of Alternaria Alternata

Strain 26-8 fermentation broth has a significant inhibitory effect on the germination of conidia of the pathogen causing jujube black spot disease (Figure 9), with an inhibition rate of 66.29% on the conidial germination of the pathogen (Table 4). The diameter of the fungal colonies of *A. alternata* after treatment with strain 26-8 was 39.71 mm, and the inhibitory rate on hyphal growth was 49.76% (Table 5). This indicates that the sterile filtrate of strain 26-8 can inhibit the growth of *Alternaria alternata* hyphae (Figure 10).

Table 4. Inhibitory effect of strain 26-8 on spore germination of *Alternaria alternata.*

Treatment	Germination rate (%)	Inhibition rate (%)
CK	88.46±2.05a	-
26-8	29.82±0.85b	66.29

[a]Means within a column followed by a common lower-case letter are not significantly different ($P>0.05$, Duncan's multiple range test).

Table 5. Inhibitory effect of strain 26-8 on hyphal growth of *Alternaria alternata.*

Treatment	Colony diameter (mm± SE) [a]	Inhibition rate (%)
CK	79.04±0.62a	-
26-8	39.71±0.31b	49.76

[a]Means within a column followed by a common lower-case letter are not significantly different ($P>0.05$, Duncan's multiple range test).

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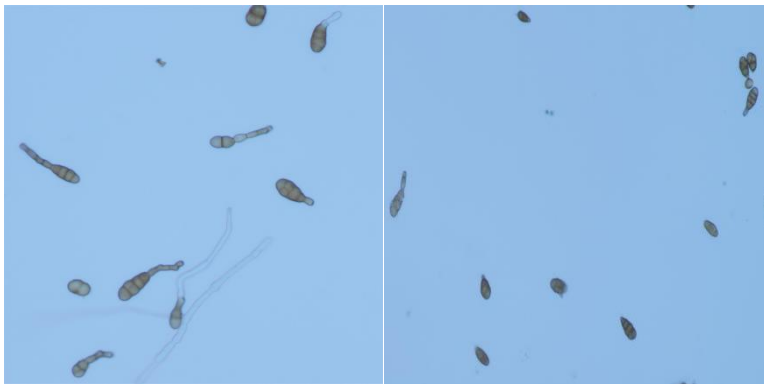

Figure 9. MICROSCOPY OF STRAIN26-8 INHIBITION OF CONIDIUM GERMINATION OF *ALTERNARIA ALTERNATA* . (UNDER 10 × 40 TIMES OPTICAL MICROSCOPE). SCALE BARS: CONTROL, 26-8= 10 ΜM.

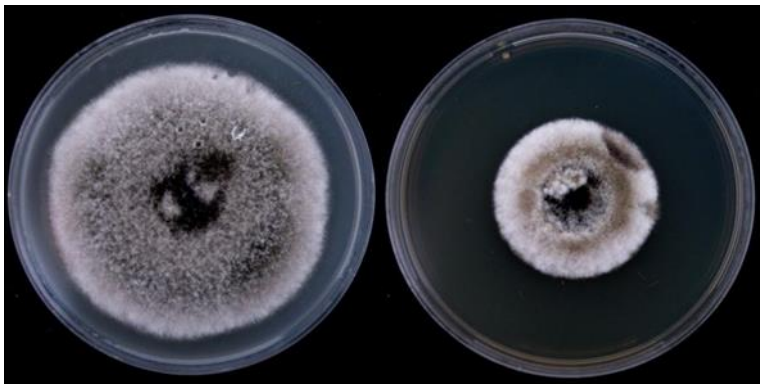

Figure 10. Diagram of inhibitory effect of antagonists on hyphal growth of Alternaria alternata.

3.5. Evaluation Results of STRAIN biocontrol Ability

Pre-inoculate the chain spore suspension on jujube fruits, inoculate the antagonistic bacteria 1 day later, and observe every 3 days. It can be seen that the lesion diameter of the treatment group is smaller than that of the control group CONTROL. At 15 days, the lesion diameter of the treatment group was 16.60mm, significantly lower than the lesion diameter of 31.70mm in the control group Control. According to the results in Table 6, the average inhibition rate of jujube fruit black spot disease in the treatment group is 50.47%. The results indicate that the antagonist 26-8 has a good control effect on jujube fruit black spot disease.

Table 6. Effect of strain 26-8 on control of black spot of jujube fruit.

Time (d)	Spot diameter (% ± SE)[a]		Inhibition rates (%)
	Treatment group	Control group	
3	5.47±0.33a	10.95±0.77a	50.01
6	8.39±0.54a	17.07±1.55b	50.88
9	10.46±1.04a	21.33±0.61b	50.95
12	14.28 ±1.69a	31.00 ±2.61b	53.95
15	16.60 ±2.56a	31.70 ±0.86b	46.57

[a]Means within a column followed by a common lower-case letter are not significantly different ($P > 0.05$, Duncan's multiple range test).

4. Discussion

Jujube black spot disease is a fungal disease caused by *A. alternata*, which commonly occurs in southern Xinjiang, significantly affecting the development of jujube industry [22]. Biological control

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is a hot topic in the research of plant disease prevention and control. *Bacillus* is one of the most widely used biocontrol microorganisms and has broad application prospects [23–26]. As a branch of *Bacillus*, the research heat of *B. velezensis* as a biocontrol strain has gradually increased in recent years. Studies have shown that it has different degrees of inhibitory effects on a variety of plant diseases such as *Fusarium* [27–30], gray mold [31–34], cotton *Verticillium* wilt [35,36]. The rapid and accurate identification of strains is the basis for subsequent development and utilization. The identification of *Bacillus* species cannot be completely and accurately distinguished from phenotypic, physiological and biochemical characteristics. With the rapid development of molecular biology technology, the identification at the molecular level has begun to be combined. The 16S rDNA gene sequence is widely used for the classification and identification of bacteria. For example, Cui Lingxiao et al. identified *Bacillus velezensis* 8-4 through 16S rDNA gene sequence analysis [37], but for closely related groups, 16S rDNA sequence analysis will fail, resulting in inaccurate identification results [38]. In recent years, researchers have found that using gene-encoded protein gene sequence as a molecular identification can make up for the deficiency of 16S rDNA gene sequence, such as gyrA, gyrB, rpoB gene and so on [20,39,40]. For example, Feng et al. analyzed and identified the strain FY-C by combining 16S rDNA and gyrB gene sequences to ensure the accuracy of the identification results [41]. In this study, the strain 26-8 was analyzed by morphological, physiological and biochemical characteristics and 16S rDNA, gyrB, rpoB gene sequences, and the strain 26-8 was identified as *Bacillus velezensis*. This study investigated the growth characteristics of *B. velezensis* 26-8, laying the foundation for the development and utilization of biocontrol strains. Strain 26-8 can grow at temperatures ranging from 24-40°C (optimal growth temperature at 28°C), pH levels from 4.0-9.0 (optimal pH at 7.0), and salt concentrations from 0.5% to 10%. This is similar to the research results on *B. velezensis* [42–44]. However, it differs from the research by Yang Di et al., where the optimal pH for *Bacillus velezensis* was 5.5 [15]. Due to the vast land area in our country with differences in soil and geographical environments, the growth and reproduction of microorganisms are also affected, resulting in different growth characteristics. Therefore, screening for a strain with stronger environmental adaptability is of great significance for biocontrol research. *B. velezensis* 26-8 has good salt and alkali tolerance, demonstrating great biocontrol potential and serving as a valuable biocontrol microbial resource.

Research has shown that *B.velezensis* can produce a variety of antibacterial metabolites, such as lipopeptides, proteins, polyketide compounds. Two entophytic antagonistic bacteria, *B.velezensis* ZJJDZY and ZJJDYB showed significant antagonistic effects against various pathogenic fungi causing black spot disease, which has the potential as biocontrol resource [45]. The control effect of *B.velezensis* GUAL210 on Rose black spot was as high as 60.96 %, and GUAL210 has promising prospects for application and development, and may be a good substitute for chemical control agents [44]. This study found that the sterile filtrate of strain 26-8 can inhibit the growth of *A. alternata*. It is speculated that one of the ways strain 26-8 exerts its biocontrol effect is by producing certain active substances to inhibit the growth of the pathogen, while further research is needed on the specific antibacterial substances and biocontrol mechanisms. A strain of *B. velezensis* isolated in this experiment showed good inhibitory effect on jujube black spot disease, with an inhibition zone diameter of 25.37mm in the plate confrontation test, and a control effect of 50.47% in the detached fruit treatment experiment. The control effect in this experiment differs from that of others, such as the study by Song C et al., which showed that when *Bacillus* amyloliquefaciens K5-1 was inoculated first on jujube fruits, the control effect on winter jujube black spot disease could reach 78.50% [46]. This may be because in this experiment, the pathogen was inoculated first followed by the antagonist, with the pathogen occupying a favorable niche first, leading to a lower control effect compared to inoculating the antagonist first. Research on biocontrol agents should not be limited to laboratory conditions, but should be able to truly apply to practical situations. Field efficacy experiments are needed to stabilize the effectiveness in the field before further development as biological agents.

5. Conclusions

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Biological characteristic experiments showed that strain 26-8 could grow at NaCl concentrations of 0.5%-10% and pH 4.0-9.0. The biocontrol experiment results showed that *Bacillus velezensis* 26-8 could achieve an 89.83% control effect against black spot disease. In conclusion, strain 26-8 has good salt and alkali tolerance, exerts a good control effect on jujube black spot disease, and is worthy of further study.

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