Isolation and identification of *Bacillus subtilis* strain T-3 from Soybean and its antagonism against several common pathogenic fungi

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Abstract. An antagonistic bacteria strain was isolated and purified from soybean using *Alternaria alternata* as indicator. Thereafter, based on morphological observation and 16S rDNA sequencing analysis, the antagonistic bacteria was identified as *Bacillus subtilis*. The culture solution of this strain showed inhibition rates of 73.19% against *A. alternata*. The strain also displayed strong antagonistic action against *Botrytis cinerea, Fusarium graminearum, Lasiodiplodia theobromae, Botrytis erythraea, Mucor circinelloides* and *Aspergillus niger*.

1. Introduction

*Alternaria* is a ubiquitous fungal genus. It is a member of *Alternaria* which belongs to the genus Pleosporaceae [1]. As a plant pathogenic fungus, it can infect a variety of fruits, vegetables and economic crops [2], causing huge losses to agricultural production. In addition to causing crop diseases, *A. alternata* can easily cause mildew in agricultural products, leading to spoilage and deterioration of vegetables and fruits [3]. Chemical fungicides such as benomyl, imazalil, carbendazim, etc. [4] are widely used in the prevention and treatment of *A. alternata* fungal diseases due to their advantages of high efficiency sterilization and significant effect. However, the long-term use of chemical fungicides will bring about safety, environmental protection and drug resistance problems. Research by the National Academy of Sciences has shown that the use of fungicides has caused significant harm to human health and environmental safety [5]. Most fungicides have a pungent odor, and are easy to cause pollution to the ecology. They also have the disadvantage of not being easily decomposed. Therefore, it is increasingly urgent to explore low-toxic and effective new environmental protection fungicides.

Biological control is a new method for the prevention and treatment of post-harvest diseases of fruits and vegetables. It has gradually become a research hotspot for fruits and vegetables preservation at home and abroad due to its safety, greenness, environmental protection, and other characteristics [6]. *Bacillus* is a type of Gram-positive bacteria which is widespread in nature. It is a kind of ideal biocontrol microorganism [7,8]. A large number of studies have shown that *Bacillus* can effectively inhibit the postharvest diseases caused by *A. alternata* on fruits and vegetables, such as peach [9], strawberry [10], pepper [11] and so on. In this study, a strain of *Bacillus subtilis* had been isolated from soybean. It was identified by morphological observation and 16S rDNA sequencing analysis method. And the antifungal effect of this *Bacillus subtilis* on *A. alternata* was determined. The results are expected to provide reference for the study of biocontrol agents for *A. alternata*.
2. Materials and Method

2.1 Experimental Materials

2.1.1 Experimental ingredients. Soybean (Commercially available).

2.1.2 Experimental medium and strain source. (1) potato dextrose agar medium (PDA): potato dextrose agar medium was weighed 46.0 g, added to 1000 ml of distilled water, autoclaved at 115 °C for 20 minutes, cooled and placed in a refrigerator at 4 °C for use. PDB medium is the liquid state of PDA medium. (2) Nutritional Agar (NA): 33.0g of nutrient agar medium was weighed 1L of distilled water, heated and boiled until completely dissolved, then dispensed and autoclaved at 121 °C for 15 minutes. Nutrition Broth is the liquid state of Nutritional Agar.

2.1.3 Experimental fungi. A. alternata, Botrytis cinerea, Fusarium graminearum, Lasiodiplodia theobromae, Botrytis erythraea, Mucor circinelloides and Aspergillus niger. These 7 strains of fungi were deposited in the laboratory of the Institute of Processing and storage of agricultural products, Chengdu Academy of Agriculture and Forestry Sciences.

2.2. Experimental method

2.2.1 Isolation of Bacillus. Soybean (25 g) was weighed and put in a triangular flask containing 225 mL of sterile saline and more than 10 glass beads, shaken thoroughly. The $10^4$ dilution suspension was prepared by centrifuge at 5000 r/min for 5 min at 4°C with a refrigerated centrifuge. The dilution suspension was heated at 80°C for 20 min in water bath and diluted to $10^6$ with sterile saline. 100 μL diluent of $10^3$ to $10^6$ were taken to NA plates by a pipette and incubated in a 37 °C constant temperature incubator for 24 hours [12]. Single colonies of different shapes were picked and streaked on the NA plate and incubated at 37 °C for 24 h for isolation and purification. The purified strains were transferred to the NA slope and placed in a refrigerator at 4 °C for future use.

2.2.2 Screening of antagonistic Bacillus. The Bacillus with an antagonistic effect on the growth of A. alternata were screened with the plate confrontation method [13]. A. alternata was inoculated on PDA plates and cultured at 25°C for 3 days. A 6 mm hole punch was taken on the plate to inoculate the fungus mass. It was inoculated to the center of the blank PDA plate, and the isolated bacilli were inoculated at the place that 15-20mm away from the fungus mass. By observing whether the growth of A. alternata mycelium was inhibited, the Bacillus with antagonistic effect was screened out under the condition of 28 °C for 5 days with non-inoculated PDA plates as blank control.

2.2.3 Determination of antifungal activity of antagonistic Bacillus culture medium. (1) Culture fluid preparation: The selected antagonistic Bacillus was inoculated into 20 mL NB medium, and cultured at 37 °C, 120 r/min for 18 hours. 1 mL of fungus solution was inoculated into 100 mL of PDB medium and incubated at 30 °C, 120 r/min for 90 h. The obtained culture solution was centrifuged at 4 °C, 10000 r/min for 20 min, the supernatant was collected, filtered through a 0.22 μm microporous filter membrane and stored at 4 °C for later use [3]. (2) The mycelial growth rate method [4] was used to determine the antifungal activity of A. alternata with the culture solution of antagonistic Bacillus. 1 mL of the microfiltration-filtered culture liquid and 19 mL of PDA medium were taken into a petri dish with the same amount of sterile PDB medium as a blank control. Each treatment has 3 duplicates. After the medium was solidified, take the A. alternata according to the method of 2.2.3. The diameters of the colony were measured by the cross method to calculate the inhibition rate under the condition of 28 °C for 7 days.

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\text{Inhibition rate} = \frac{C - T}{C} \times 100\%
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2.2.4 Determination of the inhibitory spectrum of antagonistic Bacillus. With reference to the method of 2.2.2, the bacteriostatic effect of antagonistic Bacillus against B. cinerea, F. graminearum, L. theobromae, B. erythraea, M. circinelloides and A. niger were determined.

2.2.5 Observation on morphology of antagonistic Bacillus. The purified antagonistic Bacillus was streaked on the NA plate and incubated at 37 °C for 24h. And the colony shape, color, transparency, surface, etc. were observed. A single colony was picked for Gram staining and observed the microscopic morphology of the bacteria under a 1000x oil microscope.

2.2.6 Antagonistic Bacillus 16S rDNA sequencing analysis. The selected antagonistic Bacillus strain was activated on NA plates, cultured at 37° C for 18 h. And then, single colonies were picked with a sterile inoculating ring and placed in a 3 mL nutrient broth medium, 37 ° C, 120 r/ min for 1 night. The total DNA was extracted as a template, and PCR amplification was carried out using a universal primer (27F: 5’T-AGAGTTTGATCCTGGCTCAG-3’; 1492R: 5’-TAGGGTTACCTTGATGACTT-3’) for 16Sr DNA gene. The PCR reaction system and reaction conditions refer to the methods of Liu Shaona et al. [17], and the amplified products were detected by 1.0% agarose gel electrophoresis. The PCR amplified products were recovered and sent to Shanghai Biotech Co., Ltd. for sequencing. The sequencing result was analyzed online by BLAST in NCBI. The measured gene sequence was compared with 16S rDNA gene sequences of related species in the GenBank database. Sequence analysis was performed using MEGA 7.0 software to build a phylogenetic tree.

3. Results and Discussion

3.1 Isolation and Screening of Antagonistic Bacillus
Using 5 parts of soybean as raw materials, 10 strains of Bacillus were isolated. A. alternata was used to be the indicator fungus. One Bacillus strain with a strong antagonistic effect on the growth of A. alternata was screened with the flat-panel confrontation method. The antagonistic effect of antagonistic Bacillus against A. alternata is shown in Figure 1.

![Figure 1. Antagonistic effect of T-3on A. alternata](image)

3.2 Antagonistic antifungus activity of Bacillus culture medium
As shown in Figure 2, the antagonistic Bacillus culture solution has a good inhibitory effect on the growth of A. alternata. The inhibition rate of A. alternata hypha reached 73.19% under the condition of 5% of the integral of the culture liquid. Compared with the control, the A. alternata mycelium on the plate was significantly weakened. On the back of the plate, the colony growth is dark brown after being inhibited. The results indicated that the antagonistic Bacillus culture solution can achieve the bacteriostatic effect by inhibiting the growth of mycelia of A. alternata.
Figure 2. T-3 culture solution on A. alternate (a: A. alternata + culture solution treatment, b: A. alternata blank control group, both a and b were the positive of plates; c: A. alternata + culture solution treatment, d: A. alternata blank control group, both c and d were the reverse of the tablet)

3.3 Antagonistic spectrum of Bacillus

6 common pathogenic fungi such as B. cinerea were taken as indicator fungi. The antifungal properties of antagonistic Bacillus were determined in a recent step. The results are shown in Figure 3. In addition to A. niger, the antagonistic Bacillus has different degrees of bacteriostatic effect on the remaining five fungi.

3.4 Morphological observation of antagonistic Bacillus

As shown in Figure 4a, single colonies of antagonistic Bacillus grown on nutrient agar medium is round-shaped, with irregular edges, grayish white and opaque. It can be seen from Figure 4b that the bacterium is positive for Gram. The spores which are short rod-shape grow in the middle of bacteria.

Figure 3. Antagonistic Bacillus bacteriostatic spectrum (a: B. cinerea; b: F. graminearum; c: L. theobromae; d: B. erythraea; e: M. circinelloides; f: A. niger)
3.5 Molecular Biological Identification of Antagonistic Bacillus

According to the rule that strains with 16SrDNA gene sequence similarity greater than 97% belong to the same species, the antagonistic Bacillus and Bacillus subtilis are the most recent, with a homology rate of 100%. The Bacillus is identified as Bacillus subtilis. The phylogenetic tree is shown in Figure 5.

4. Conclusions

The Bacillus subtilis screened in this study is expected to be a strain developed as a preservative for microbial sources. It has prevention and treatment effect on A. alternata, B. cinerea, F. graminearum, L. theobromae, B. erythraea, and M. circinelloides. The growth of these myceliums were inhibited. It provides a certain theoretical basis for the preparation of natural, environmentally friendly and safe biological fungicides. The application of this Bacillus subtilis is expected to reduce the use of chemical agents in modern agricultural systems.

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