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Screening, Isolating, and Identifying Antagonistic Bacterium from the Rhizosphere Soil of *Alternaria* Brown Spot

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Abstract. *Alternaria* brown spot is a serious agricultural fungal disease that consistently leads to serious economic losses in commercial crop fields worldwide. Antagonistic bacteria were isolated from 131 mature-period soil samples from eight counties in Bijie City, Guizhou Province, China. In this study, the antagonistic bacteria that inhibited *Alternaria alternata* were screened and isolated. In addition, the antagonistic strain L2 was identified as *Bacillus megaterium*.

1. Introduction

Alternaria brown spot [1] is caused by the genera *Alternaria*, controlling the disease has been a contested issue owing to the potentially negative environmental consequences associated with combating the pathogen. The environmental pollution caused by the broad application of chemical fungicides has become an increasing concern in agricultural production. One such possibility is utilizing microorganisms [2]. Since Lorenz Hiltner elaborated upon the rhizosphere concept in 1904, many studies have reported that the soil environment attached to the root system is a hot spot of biocontrol [3, 4]. Consequently, in this study, the target bacteria were isolated from the rhizosphere soil of infected plants.

2. Materials And Methods

2.1. Materials

Alternaria alternata, was isolated and preserved by The Institute of Fungi Resource, College of Life Science, Guizhou University.

2.2. Culture media

1) Beef extract-peptone medium: 5 g/L beef extract, 10 g/L peptone, 5 g/L NaCl, 15–20 g/L agarose, pH 7.0–7.2, sterilized at 121°C for 30 min.

2) Potato dextrose agar medium (PDA): 200 g/L potato, 20 g/L dextrose, 15–20 g/L agarose, and natural pH, sterilized for 30 min at 121°C.



3) Dextrose fermentation liquid medium: 5 g peptone, 5 g NaCl, 0.2 g K_2HPO_4 , 10 g dextrose, and 3 mL of 1% bromothymol blue, in 1 L of distilled water, pH 7.0–7.4, sterilized at 121°C for 20 min.

4) V-P liquid medium: 5 g peptone, 5 g dextrose, and 5 g NaCl, in 1 L of distilled water at pH 7.0–7.2.

5) Starch medium: 0.2% soluble starch, 5 g/L beef extract, 10 g/L peptone, 5 g/L NaCl and 15–20 g/L agarose at pH 7.0–7.2.

6) Simmons citrate medium: 2 g sodium citrate, 5 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g $K_2HPO_4 \cdot 3H_2O$, 1 g NH_4HPO_4 , 10 mL of 1% bromothymol blue, 15–20 g of agarose, and 1 L of distilled water.

7) Litmus milk medium: Approximately 10 g of skimmed milk powder was dissolved into 100 mL of distilled water. Additionally, 1 g of litmus was soaked in 40 mL of distilled water overnight or longer and fully softened. Then, 4 mL of the litmus solution and 100 mL of the skimmed milk powder solution were fully mixed until the mixture turned a lilac purple. The mixture was then sterilized at 115°C for 20 min.

8) Gelatin liquefaction medium: 5 g peptone, 140 g gelatin, and 1 L of distilled water at pH 7.2–7.4.

2.3. Methods

2.3.1. Soil samples collected. Plough-layer soils were collected from both normal and *A. alternata*-affected tobacco-growing fields throughout eight counties in Bijie City, Guizhou Province. The non-soil parts, including gravel, were removed from the samples. The sampling depth was 3–10 cm underground.

2.3.2. Separation, purification, and storage of the soil bacteria. Approximately 10 g of tobacco-growing soil was added to 90 mL of sterile water containing glass beads. The mixture was oscillated in a shaking bed at 160 rpm for 20 min. After 5 min of standing, the suspension was removed by suction and gradient diluted with sterile water. Then, 0.1 mL of the diluent was removed using suction and brushed onto a beef extract-peptone plate. After 36 h of inverting the culture at 37°C, Then, single colonies were selected, marked, purified, and stored until testing.

2.4. Screening of the antagonistic bacteria against brown spot

Preliminary screening: Using the plate confrontation method [5], *A. alternata* pellets were removed from the PDA medium with a 0.5-cm hole puncher and placed at the centers of prepared PDA plates. Then, the testing strains were symmetrically inoculated around the center at a distance of 2.5 cm. A non-inoculated plate functioned as the control. Each test was conducted in triplicate. After cultivating for 5 d in a 28°C incubator, the formation of inhibition zones among the test groups was observed, and, if present, the zone's size was measured. Strains from the preliminary screening that had inhibition zones with diameters greater than 1.0 cm were transferred into triangular flasks that contained the beef extract-peptone solution. After 2–3 d of shaking at 150 rpm/min at 28°C, the fermentation broths were used to measure antagonistic effects.

Second screening: Using a broth-filter paper method, *A. alternata* pellets were removed from the PDA medium with a 0.5-cm hole puncher and placed at the center of the prepared PDA plates. Three pieces of filter paper (0.5-cm diameters) soaked with L2 fermentation broth were placed symmetrically around the center at a distance of 2.5 cm. A control was prepared using filter paper that had not been immersed in the fermentation broth. Each test was conducted in triplicate. After cultivating for 5 d in a 28°C incubator, the sizes of the inhibition zones were measured.

3. Strain identification

3.1. Morphological, culture, and physiological-biochemical characteristics

Using a plate-streak method, the strains were inoculated onto plated media. After culturing for 24 h at 37°C, the morphology was observed using regular methods.

The physiological-biochemical characteristics were identified as per the Common Bacteria Identification Manual, Bergeys' Manual of Systematic Bacteriology (Edition 9).

3.2. 16S rDNA sequencing

The strain L2 was inoculated into the beef extract-peptone media and cultured at 160 rpm at 28°C for 12 h, followed by centrifugation to collect the cells. The genomic DNA was extracted using an Ezup columnar-type genome DNA extraction kit (for bacteria, Shanghai Sangon Biotech Co.). Using the extracted DNA as a template, the 16S rDNA genes were PCR-amplified with the forward 16SF primer (5'-GAGAGTTTGATCCTGGCTCAG-3') and the reverse 16SR primer (5'-AAGGAGGTGATCCAGCCGCA-3'). The PCR conditions were set as follows: at 94°C for 3 min, 25 cycles of 94°C for 45 s, 54°C for 30 s, and 72°C for 1 min, followed by 72°C extension for 5 min, with a 4°C termination. PCR products were detected using 1% agarose gel electrophoresis, staining, and gel imaging, and then, the PCR products were sent to Shanghai Sangon Biotech for sequencing. The obtained sequence was compared with those on NCBI using a BLAST algorithm-based search of colonial morphology, physiological-biochemical properties, and molecular identification.

4. Results And Analysis

4.1. Screening and separation of antagonistic bacteria

The soils were separated using a dilution-plate method. A total of 164 strains of bacteria were separated from the 131 soil samples. The bacteria were purified using a plate-streak method. The separated strains were mixed with 15% glycerol and then stored in bacterial preservation tubes for later testing. After the preliminary screening, 18 strains had antagonistic effects, especially strain L2, which had an inhibition zone with a diameter > 1.01 cm (Fig. 1A). A second screening (Fig. 1B) showed that the fermentation broth from strain L2 significantly inhibited *A. alternata* mycelial growth, with an average inhibition diameter of 1.12 cm ± 0.012.

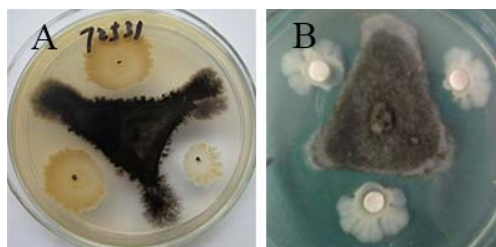


Fig. 1 Antagonistic strain L2 of tobacco *Alternaria alternata*
A. Effect of strain L2 on spore-bacterial plates of *A. alternata*
B. Effects of strain L2's fermentation broth on *A. alternata*.

4.2. Identification of the strain

The antagonistic strain L2 was inoculated onto a PDA plate and cultured at 37°C for 36 h. The fungal colonies were nearly round, convex, yellow, and nontransparent. Moreover, these colonies were 0.2–0.4 cm in size with orderly margins and either bright or dark surfaces, and they did not secrete pigments. These Gram positive bacteria were rod-like, 1.2–1.5 × 2.0–4.0 μm in size and arranged in a chain-like way. The spores were elliptical, terminal, or semi-terminal, but they did not obviously swell.

Physiological-biochemical tests (Table 1) showed that strain L2 could tolerate 7% NaCl. Strain L2 tested positive in the following tests: catalase, dextrose fermentation, starch hydrolysis, nitrate reduction, gelatin liquefaction, semisolid puncture, milk decomposition, citrate utilization, and pH 5.7 growth. However, it tested negative in the V-P, indoles, and mannitol hydrolysis tests.

Table 1. Strain L2's morphology, and physiological and biochemical characteristics

Project	L2 strain
<u>Surface</u>	<u>Hump</u>
Colony morphology	Small single colony
Colony color	White/Cream
Thallus morphology	Short rod
Diaphaneity	Opacification
Gram stain	+
Moveability	<u>Active</u>
2 %NaCl	+
5 %NaCl	+
7 %NaCl	+
10 %NaCl	-
Starch hydrolysis	+
Gelatin hydrolysis	+
Milk decomposition	+
Sugar fermentation	+
V-P test	-
Citrate test	+
Nitrate reduction test	+
<u>Indole test</u>	-
<u>Catalase test</u>	+
pH5.7	+
Semi solid puncture	+
Mannitol hydrolysis	-

Note: "+"positive; "-"negative

When using the genomic DNA of L2 as the template, PCR-amplified products were detected through 1% agarose gel electrophoresis, which generated a 1.5-kb specific band (Fig. 2). The full-length of L2 was 1,471 bp, as determined by 16S rDNA sequencing. The maximum homogeneity was compared between this sequence and other relevant sequences in GenBank, and strain L2 shared 99% homogeneity with *B. megaterium*.

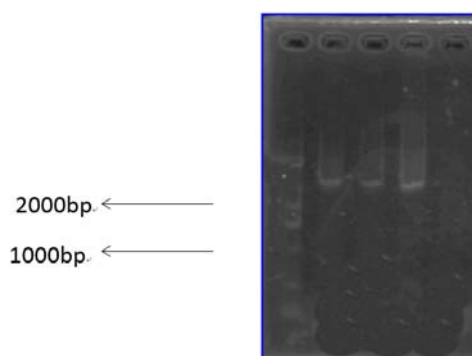
**Fig 2.** L2 PCR amplification products.

Fig. 2 Details of the genetic relationship between L2 and *Bacillus megaterium* strain ATCC14581 and L2 and *Bacillus aryabhattai*. Strain B8W22. However, *B. aryabhattai*'s colony was peach in color, which distinguished it from L2. Therefore, based on the morphological, physiological-biochemical, and molecular biotechnical tests, we identified the antagonistic strain L2 as *B. megaterium* strain L2.

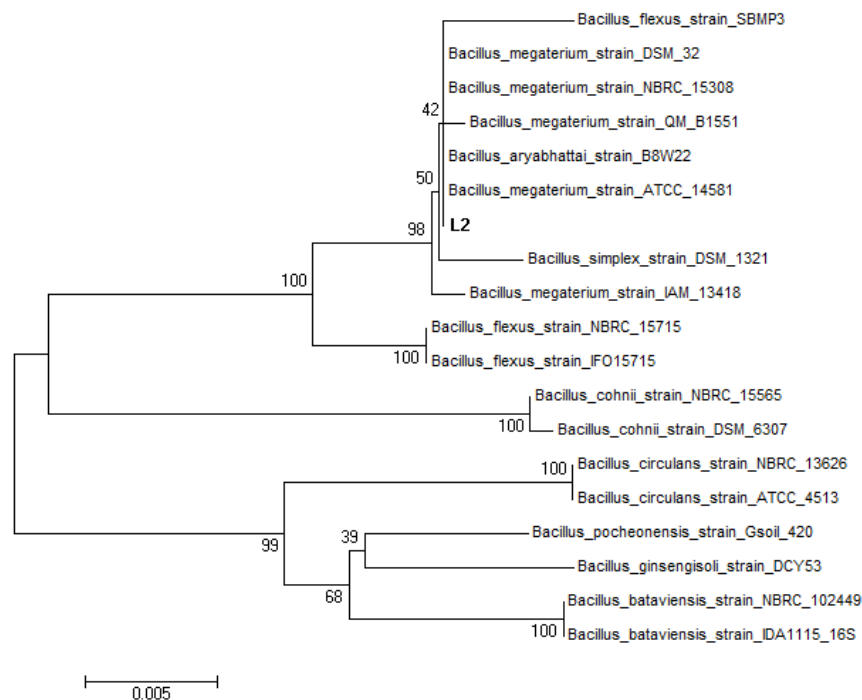


Fig. 3 Neighbor-joining phylogenetic tree

Fig. 3 shows the neighbor-joining phylogenetic tree constructed using 16S rRNA gene sequences of strain L2 and closely related species of the genera *Bacillus*. The numbers at the nodes are the percentages that indicate the level of bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets. The scale bar represents 0.005 substitutions per nucleotide position.

Acknowledgements

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