Isolation and Characterization of Bacillus amyloliquefaciens TL6 as a Biological Control Agent for Peanut Early Leaf Spot Caused by Passalora arachidicola

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Isolation and Characterization of *Bacillus amyloliquefaciens* TL6 as a biological control agent for peanut early leaf spot caused by *Passalora arachidicola*

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Abstract

Peanut early leaf spot caused by *Passalora arachidicola* (*Cercospora arachidicola*), is a worldwide common fungal disease in peanut leaves, which occurs in all production areas in China. To obtain biocontrol resources to control peanut early leaf spot, 60 healthy peanut leaves were collected from major peanut production areas in Liaoning Province. A total of 563 strains were screened from these leaves. Eighteen strains showed differing levels of resistance against *P. arachidicola*. Among these strains, strain TL6 inhibited the pathogen most strongly, and the diameter of inhibition zone was 64.3 mm. This strain was able to inhibit 10 other types of pathogens. It was identified as *Bacillus amyloliquefaciens* based on its morphological characteristics, physiological and biochemical reactions and a comparative analysis of its 16S rDNA sequence. The fermentation liquor of strain TL6 was effective at controlling peanut early leaf spot, and the field control effect was above 69.17% after spraying the fermentation liquid of TL6. The field control effect was more than 40.96% after spraying the fermentation liquid diluted 200 times. The field control effect of the TL6 fermentation liquid diluted 200 times and including the addition of 500 $g \cdot L^{-1}$ carbendazim diluted 1000 times inhibited *P. arachidicola* by 81.33%. The combination of TL6 and carbendazim had a significant synergistic effect. This strain of *B. amyloliquefaciens* shows promise for commercial development and application.

Peanut is an important economic and oil crop[1]. China is the largest peanut producer in the world[2]. The average annual output of peanuts is 17 million t in China，accounting for about 40% of the world's total peanut output; and the annual export is 700 000 t，

accounting for 47% of the world's total trade in peanuts[3]. Liaoning is a province with large plantings of peanuts. In recent years, the average annual area in Liaoning planted to peanuts has been approximately 370,000 hectares[4]. It has become the third largest cultivated crop after corn and rice. Moreover, peanuts from Liaoning Province are famous in China and throughout the world for their good quality and taste and lack of aflatoxins[5].

Peanut early leaf spot, caused by *P arachidicola* (*C. arachidicola*), is a worldwide common fungal disease in peanut leaves, which occurs in all production areas[6]. The disease occurs in the early and middle stage of peanut growth and primarily damages the leaves. When the disease occurs, a large number of round or irregularly shaped lesions appear on the leaves with an obvious yellow halo, causing early defoliation and seriously affecting the photosynthetic efficiency of plants. The yield of damaged peanuts is generally reduced by 15-59%[7].

Currently, early leaf spot in peanut production is primarily controlled by chemical pesticides[8-10]. However, the problems of resistance, resurgence and residues caused by the long-term use of chemical pesticides have become increasingly prominent[11]. Research on disease management around the world primarily focuses on biological control or the joint management of biological agents and fungicides[12]. During the past few decades, researchers had studied the antagonistic effects of a variety of fungal and bacterial biocontrol agents[13-14]. *Bacillus cereus* 304 significantly reduced the severity of early leaf spot on chitin-modified leaves[15]. It is clear that research on leaf spot disease caused by *P. arachidicola* is essential for control by plant extracts and

biological control instead of using chemicals with the goal of avoiding environmental pollution[16]. We screened microorganisms from peanut leaves to obtain strains with strong inhibitory activity. This research should lay a theoretical foundation for the development of biocontrol agents against peanut early leaf spot.

Results

Isolation and screening of biocontrol agents against *P. arachidicola*

A total of 465 microorganisms were isolated from peanut leaves, including 435 strains of bacteria, and 30 strains of actinomycetes. The results showed that 16 bacteria and six actinomycetes showed differing degrees of inhibition against *P. arachidicola* (Table 1). Among them, bacterial strain TL6 showed the strongest inhibitory effect on *P. arachidicola*, and the diameter of inhibition zone was 64.3 mm (Figure 1).

Strain number	Strain	Inhibition	Strain number	Strain type	Inhibition
SY3	bacterium	23.7±1.39 f	DD27	bacterium	35.0±1.48 c
SY23	bacterium	15.3±1.08 jk	DD35	bacterium	31.3±0.99 d
FX38	bacterium	25.0±1.61 f	CY5	bacterium	15.7±0.37 ij
TL6	bacterium	64.3±1.73 a	CY33	bacterium	19.3±0.83 g
TL22	bacterium	33.3±1.79 cd	HLD25	bacterium	18.0±0.50 ghi
TL35	bacterium	18.7±1.02 gh	HLD41	actinomycete	16.5±1.10 hij
LY18	bacterium	19.7±0.70 g	AS15	actinomycete	13.3±0.57 kl
LY55	bacterium	10.3±0.36 m	AS25	actinomycete	17.7±0.45 ghij
DD13	bacterium	11.0±0.45 lm	TL25	actinomycete	25.3±1.16 f
DD14	bacterium	12.7±0.42 l	DD38	actinomycete	28.0±1.36 e
DD18	bacterium	37.3±0.79 b	DD45	actinomycete	34.0±1.70 c

Table 1. The inhibitory effect of different isolates against *Cercospora. arachidicola*

Note: Means within the same column followed by different letters (a, b, c, d, e, f, g, h, i, j, k, l, m) are significantly different ($P < 0.05$) according to the Duncan's test.

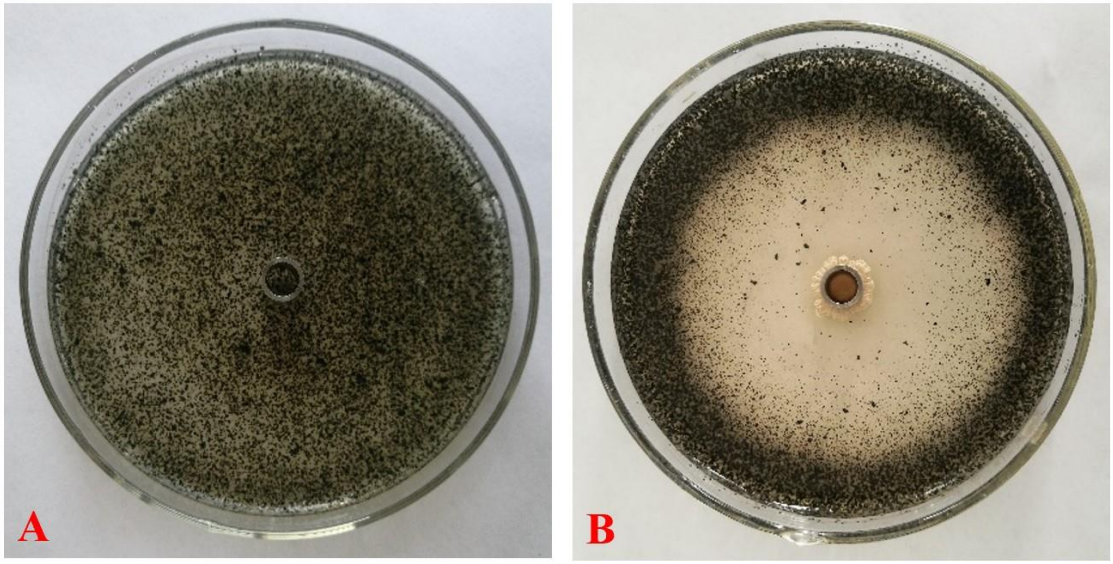

Figure 1. The efficacy of TL6 strain against *C. arachidicola* in PDA plates

A: control; B: treatment with strain TL6

Antimicrobial spectrum assay of strain TL6

Strain TL6 had differing degrees of inhibition on 10 types of plant pathogens, and the inhibitory zones were 6.2-20.3 mm. The inhibitory activity of TL6 strain against *P. arachidicola* was strong, and the inhibition zone was 20.3 mm. However, the inhibitory activity of strain TL6 against *Botryosphaeria berengeriana* was weak, and the inhibition zone was only 6.2 mm (Table 2).

Pathogens	Inhibitory diameter (mm)	Pathogens	Inhibitory diameter (mm)
Cercospora arachidicola	20.3±0.45 a	*Fusarium oxysporum*	13.4±0.65 c
Phytophthora capsici	16.7±0.32 b	*Colletotrichum orbiculare*	12.8±0.36 c
Coniella diplodiella	8.2±0.78 f	*Botryosphaeria berengeriana*	6.2±0.62 g
Phytophthora infestans	10.5±0.43 e	*Fusarium graminearum*	9.1±0.37 f
Botrytis cinerea	11.7±0.59 d	*Exserohilum turcicum*	15.8±0.29 b

Table 2. Inhibitory spectrum of strain TL6 against pathogens

Note: Means within the same column followed by different letters (a, b, c, d, e, f) are

significantly different (P <0.05) according to the Duncan's test.

Biological control efficiency of strain TL6 strain in the field

The result showed that the ability of original fermentation solution of TL6 on peanut early leaf spot were 71.34% and 69.17% respectively at 2019 and 2020, which was equivalent to the control effect of 500 $g \cdot L^{-1}$ carbendazim suspension diluted 1000 times. The control effect of the original fermentation solution of TL6 that was diluted 200 times on peanut early leaf spot were 40.96% and 42.48% respectively at 2019 and 2020. The control effect of the TL6 fermentation solution diluted 200 times + 500 $g \cdot L^{-1}$ carbendazim suspension diluted 1000 times were 84.67% and 81.33% respectively at 2019 and 2020, which was significantly higher than that of either treatment used alone (Table 3).

Treatment	2019		2020	
	Average disease	control effect	Average disease	control effect
A1	3.61	71.34±2.23 bc	3.65	69.17±1.93 c
A2	5.91	53.14±2.14 d	5.32	55.07±4.09 d
A3	7.02	44.35±2.49 e	6.67	43.67±2.13 e
A4	7.44	40.96±1.60 e	6.81	42.48±0.88 e
B	3.27	74.08±2.68 b	3.25	72.55±2.16 bc
C	1.93	84.67±1.39 a	2.21	81.33±1.41 a
D	12.61	-	11.84	-

Table 3. The control effect of stain TL6 against *Cercospora arachidicola* in the field

Note: Means within the same column followed by different letters (a, b, c, d, e) are significantly different (P <0.05) according to the Duncan's test.

Characterization of strain TL6

The physiological and biochemical tests showed that the strain TL6 was Gram-positive. It could grow in less than 10% NaCl solution and at 5-45℃. The results of the oxidase reaction, milk hydrolysis, starch hydrolysis, gelatin liquefaction,

nitrate reduction, citrate utilization, contact enzyme reaction and methyl red test were positive, while the hydrogen sulfide and V-P reaction were negative (Table 4).

physiological and biochemical	result	physiological and biochemical	Result
Gram staining	+	V-P reaction	-
Salt tolerance	≤ 10%	Gelatin liquefaction test	+
Growth temperature	5-45 ℃	Nitrate reduction test	+
Oxidase test	+	Citrate utilization test	+
Milk hydrolysis	+	Contact enzyme reaction	+
Hydrogen sulfide generation test	-	Methyl red test	+
Starch hydrolysis test	+		

Table 4. The physiological and biochemical characteristics of strain TL6

Note: + indicates a positive reaction. - indicates a negative reaction.

The sequencing results showed that the 16S rDNA of TL6 strain was 1420 bp. A BLAST homologous sequence analysis in NCBI showed that strain TL6 was similar to the type strain of *Bacillus amyloliquefaciens* ATCC 23350 (Figure 2). Based on its physiological and biochemical characteristics and 16S rDNA gene sequence, strain TL6 strain was identified as *B. amyloliquefaciens*. The 16S rDNA sequence of isolate TL6 was deposited in the GenBank (MW548727.1).

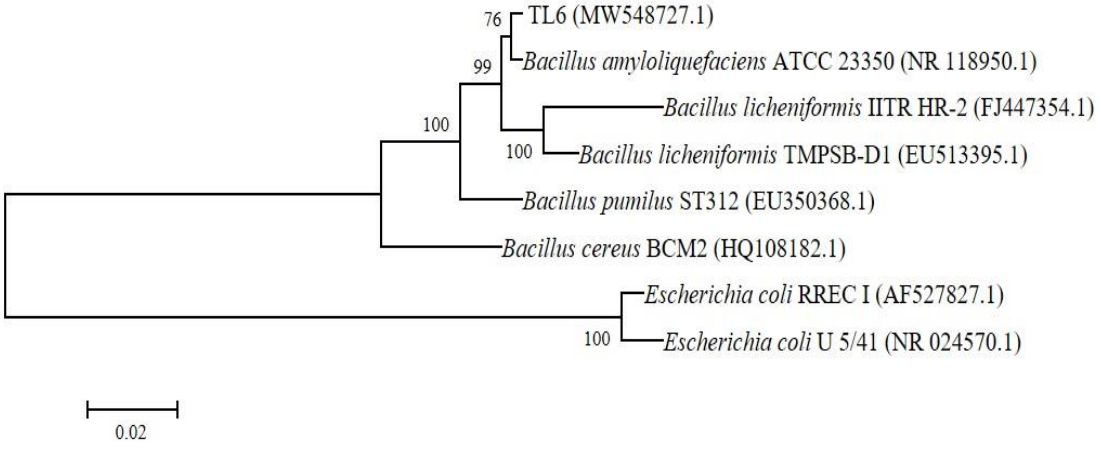

Figure 2. A phylogenetic neighbor-joining tree showing the relationship of the TL6 strain with other related species. Bootstrap values of 100 analyses are shown at the branch points. The scale bar represents two nucleotide substitutions per 100

nucleotides of 16S rDNA sequence.

Discussion

Early leaf spot is one of the serious diseases that causes substantial economic losses in peanut production. The current control measures primarily rely on the selection of resistant varieties and use of chemical controls. Biological control is considered as one of the most potential control methods for its pollution-free and long-term effects [17]. Extensive research on the development and utilization of *Bacillus* for biological control have been conducted in China and throughout the world[18]. Currently, there are 110 products registered in China for the control of plant diseases and insect pests (China Pesticide Information Network. http://www.chinapesticide.org.cn/). However, there are no reports on the control of peanut early leaf spot by species of *Bacillus*. In this study, *B. amyloliquefaciens* TL6 was screened from peanut leaves, and it strongly inhibited *P. arachidicola*, and it also had different degrees of inhibitory activity against other pathogenic fungi. The control effect of the original fermentation solution of TL6 diluted 200 times against early leaf spot was more than 40.96%. There was no significant difference in the control effect of all the same treatments, which proved that the control effect of TL6 fermentation liquor was relatively stable.

Bacillus amyloliquefaciens was named by Fukomato in 1943 and was not included on the Approved Lists of Bacterial Names and has not been validly published since January 1, 1980[19]. *Bacillus. amyloliquefaciens* is found widely in the soil and on plants, fruit and vegetable surfaces, plant compost and healthy animal feces, so it was easy to screen *B. amyloliquefaciens* with antagonistic activity that

could not only inhibit the growth of plant pathogens but also promote the growth of crops and improve the number and weight of potential biocontrol agent of fruit [20, 21]. *B. amyloliquefaciens* has a broad spectrum of inhibitory activity that contains rich and diverse active substances, primarily including antagonistic proteins[22], surfactin[23], iturins[24], and chitinase[25]. These active substances play an important role in inhibiting fungi and bacteria. *B. amyloliquefaciens* BaX030, isolated from soil, showed strong antagonistic activity against *Staphylococcus aureus*, *Candida albicans*, *Saccharomycetes*, *Pyricularia grisea*, *Colletotrichum acutata* and *Phytophthora parasitica*[26]. *Bacillus amyloliquefaciens* B190 is effective at controlling lily grey mold (*Botrytis elliptica*), and the inhibitory effect was even significantly higher than the commonly used fungicides prochloraz and acetamioxime[27].

As a potential biocontrol microbial resource, *B. amyloliquefaciens* had not been used to control peanut early leaf spot. In this study, we found that *B. amyloliquefaciens* TL6 was effective at controlling *P. arachidicola* in the field, and combined with carbendazim, it had an obvious synergistic effect. Next, we could explore whether it could colonize the internal parts of peanut plants as an endophyte. On this basis, we separated and purified the antimicrobial active substances of strain TL6 and clarified the antimicrobial mechanisms. This research would provide important security for the development of corresponding biocontrol agents that are safe and effective and could serve as agents for the long-term prevention and control of peanut early leaf spot, and the reduction of chemical pesticides in peanut production.

Materials and methods

Sample collection and isolation of bacteria and actinomycetes

Sixty healthy leaves were collected from Fuxin, Shenyang, Jinzhou, Tieling and other major peanut producing areas in Liaoning Province in China. The leaves were washed with sterile water and cut into 2-3 cm segments, washed with sterile water three times and placed in a beaker. A volume of 200 mL of normal saline and 0.25 mL Tween-80 was added, vibrated with 130 $r \cdot min^{-1}$ at 25℃ for 30 min and incubated for 30 min. A volume of 1 mL of supernatant was smeared on beef extract-peptone agar and Gao's No. 1 media with a coating rod. A single colony was isolated and purified in an incubator at 27 ℃ for 48 h.

In vitro evaluation of isolates against *P. arachidicola*

The effect of the selected bacterial isolates in suppressing the growth of *P. arachidicola* was evaluated using the Oxford cup method in potato dextrose agar (PDA) medium as described by Chen[28]. A suspension of 1×10^5 spores per milliliter of *P. arachidicola* was prepared, and 5 mL of a spore suspension was added to 100 mL uncoagulated PDA medium, shaken well, and poured as plates. After the media had solidified, the Oxford cup (Φ=6 mm) was placed in the center of the PDA plate, and 0.3 mL of microbial liquid was added to the Oxford cup. The diameter of inhibitory zone around the Oxford cup was measured after incubation for 5-7 days at 27℃. Each treatment was repeated three times.

Antimicrobial spectrum assay

Isolate TL6 that had strong inhibitory activity was selected to test against other fungal

plant pathogens, including *Phytophthora capsica*, *Coniella diplodiella*, *P. infestans*, *Botrytis cinerea*, *Fusarium oxysporum*, *Colletotrichum orbiculare*, *Botryosphaeria berengeriana*, *F. graminearum* and *Exserohilum turcicum*, on PDA plates using the dual culture technique. The pathogen was placed on one side of the plate with culture medium, and TL6 strain was placed on the other side. The addition of just the pathogen to the plates served as the control. The plate was placed in incubators to culture. After incubation for 7 days at 25℃, the ability of biocontrol agents to inhibit the pathogen was observed[29].

Biological control efficiency of strain TL6 in the field

Preparation of original fermentation solution of strain TL6

Preserved strain TL6 was added to the beef extract-peptone media and activated at 28℃ for 48 h. A 1 mL suspension of 1×10^{9} CFU·mL^{-1} of strain TL6 was extracted and added to 110 mL beef extract-peptone media in a 250 mL triangular flask and then shaken at 150 r·min^{-1} at 25℃ for 96 h to obtain the original fermentation solution of strain TL6 for following text.

Biological control efficiency of strain TL6 in the field

The experiment was conducted began June 25, 2019 and June 28, 2020 respectively in the peanut experimental plot of Liaoning Academy of Agricultural Sciences, Shenyang, China. Peanuts had been cultivated on this experimental site for many years, and peanut early leaf spot occurred seriously every year. The peanut variety was Silihong. During the experiment, the peanut was only cultivated and managed normally, i.e., it was fertilized and irrigated normally, but no other chemicals were

sprayed on the crop. The experiment was designed according to the GB/T 17980.85-2004 guidelines (Pesticide--Guidelines for the field efficacy trials (II)--Part 85: Fungicides against Alternaria leaf spots of peanut)[30], with a total of seven treatments. The treatments included the original fermentation solution of TL6 strain, which was diluted 10, 100 and 200 times, a 50% suspension of Carbendazim (Jiangsu Longdeng Chemical Co., Ltd, http:// jsld.company.lookchem.cn/), 500 g/L suspension of Carbendazim diluted 1000 times + the original fermentation solution diluted 10 times (Table 5).

Letter	Treatment	Letter	Treatment
A1	original fermentation solution of TL6	B	500 g·L^{-1} Carbendazim suspension diluted 1000 times
A2	original fermentation solution of TL6 diluted 10 times	C	original fermentation solution of TL6 strain diluted 200 times +500 g·L^{-1} Carbendazim suspension diluted 1000 times
A3	original fermentation solution of TL6 diluted 100 times	D	control
A4	original fermentation solution of TL6 diluted 200 times		

Table 5. The experimental treatment

Each treatment had 30 holes of peanuts. The experimental plots were randomly arranged (Figure 3). Each treatment was repeated four times. All leaves of 10 peanut plants were randomly investigated in each plot, and the number of diseased leaves at all levels was recorded as described by Zhou et al[31]. The leaf grading method was divided into five grades according to the percentage of lesion area in the whole leaf area as follows: grade 0, no lesion; grade 1: the lesion area was less than 10% of the whole leaf area; grade 2: the lesion area was 10%-25% of the whole leaf area; grade 3: the lesion area was 25%-50% of the whole leaf area Grade 4: the area of diseased leaf

was 50%-75% of the whole leaf area; grade 5: the area of diseased leaf was more than 75% of the whole leaf area; the diseased leaves were shed, and the plant died. The control effect was calculated using a disease index.

$$DI = \frac{\sum(A \times B)}{M \times B\max} \times 100$$

$$I(\%) = \frac{Zck - Zx}{Zck} \times 100$$

Where A = Number of diseased leaves of all levels; B = the level of each diseased leaf; M = total number of leaves; Bmax = the highest level of disease; I = control effect; Zck = the disease index of control group, and Zx = the disease index of treatment group.

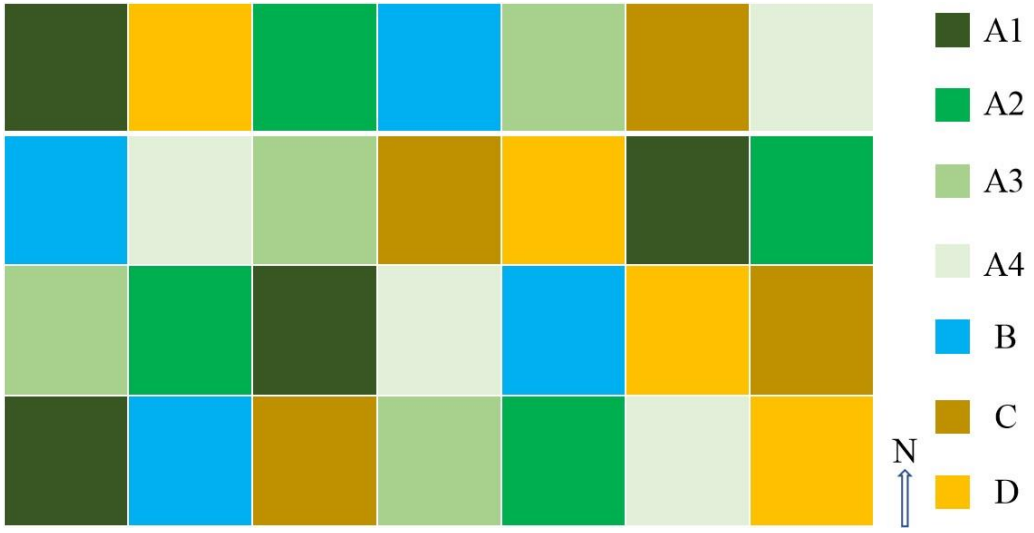

Figure 3. The layout of biological control efficiency in the field assay

A1 represents the original fermentation solution of TL6 strain; A2 is A1 diluted 10x; A3 is A1 diluted 100x; A4 is A1 diluted 200x; B is in 1000 times of 500 $g \cdot L^{-1}$ Carbendazim suspension; C is A4+B; D is the control of clear water.

Characterization of strain TL6

Physiological and biochemical tests

The physiological and biochemical tests were conducted as described by Dong[32]. The

following tests were performed on strain TL6: gram stain, salt tolerance test, growth temperature test, oxidase reaction, milk hydrolysis test, hydrogen sulfide test, starch hydrolysis test, and Voges-Proskauer (V-P) reaction among others (Table 4).

16S rDNA sequence analysis

To extract the DNA of strain TL6, the cells were harvested from 10 ml of overnight incubated culture, and the pellets were lysed in 1 mL of lysis buffer (25 % sucrose, 20 mM EDTA, 50 mM Tris-HCl and 5 $mg \cdot mL^{-1}$ of lysozyme). The chromosomal DNA was extracted as described by Zhang et al[33].

The 16S rDNA was amplified by PCR with the universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). The PCR reaction system contained 1.0 μL DNA, 2.5 μL 10×PCR buffer, 0.5 μL upstream primer (27f), 0.5 μL downstream primer (1492r), 1.0 μL dNTP, 0.5 μL Taq polymerase, and 19.0 μL ddH_2O. The amplification conditions were established for the initial denaturation at 95℃ (5 min), followed by 35 cycles at 95℃ (30 s), 57℃ (45 s) and 72℃ (1.5 min), with a final extension at 72℃ for 5 min. The PCR products were sequenced by Shanghai Sangon Biotech, China (https://www.sangon.com/). The sequences were compared using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) to identify strain TL6.

Statistical analysis

The data were subjected to analysis using one-way analyses of variance (ANOVAs). followed by Duncan's multiple means comparisons at $P < 0.05$ (SPSS 24.0, IBM, Inc., Armonk, NY, USA)

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365 **Disclosure statement**

366 No potential conflict of interest was reported by the author(s).

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Statement

The peanut or seed/leaf specimens we had been collected was complied with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora. The Experiments and research conducted on plants or collected plant material are in accordance with Chinese related legislation.

Figures

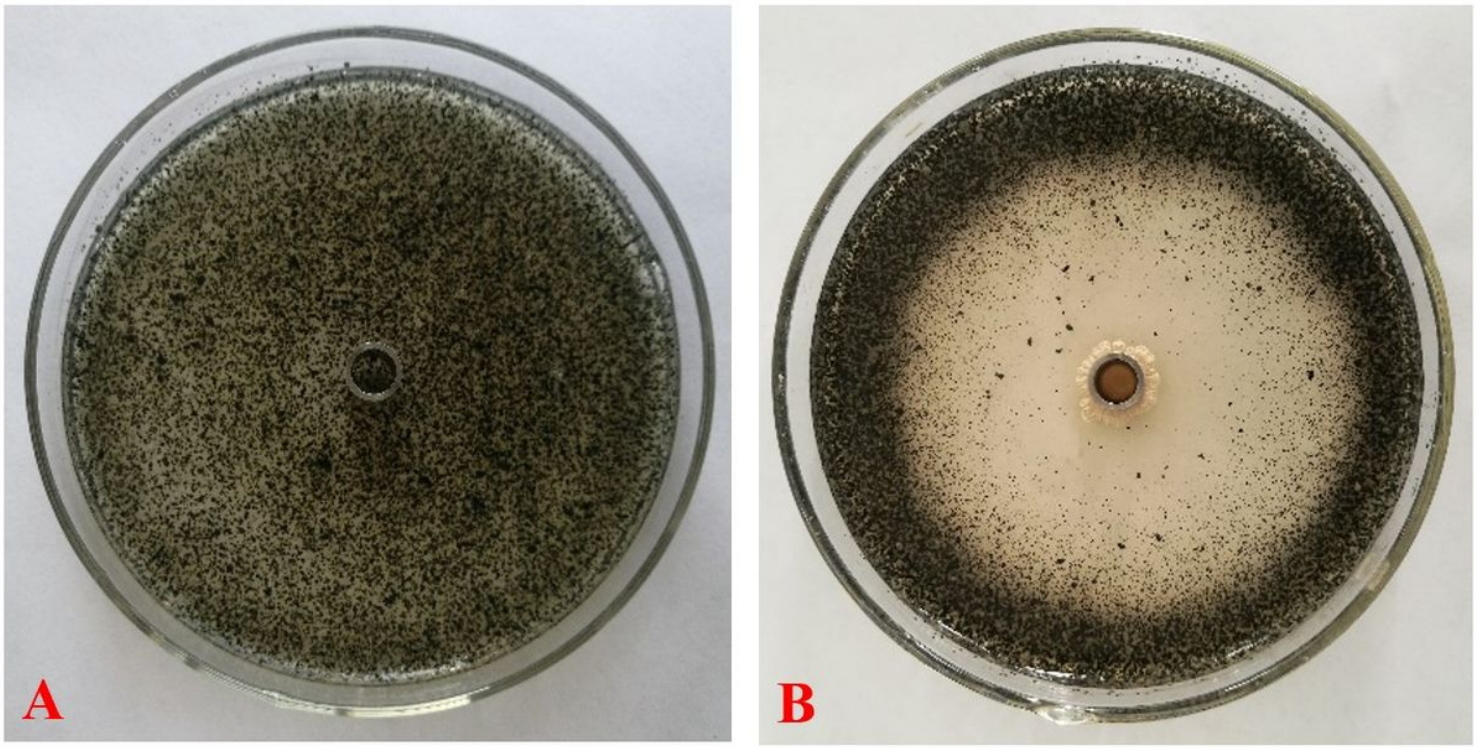

Figure 1

The efficacy of TL6 strain against C. arachidicola in PDA plates A: control; B: treatment with strain TL6

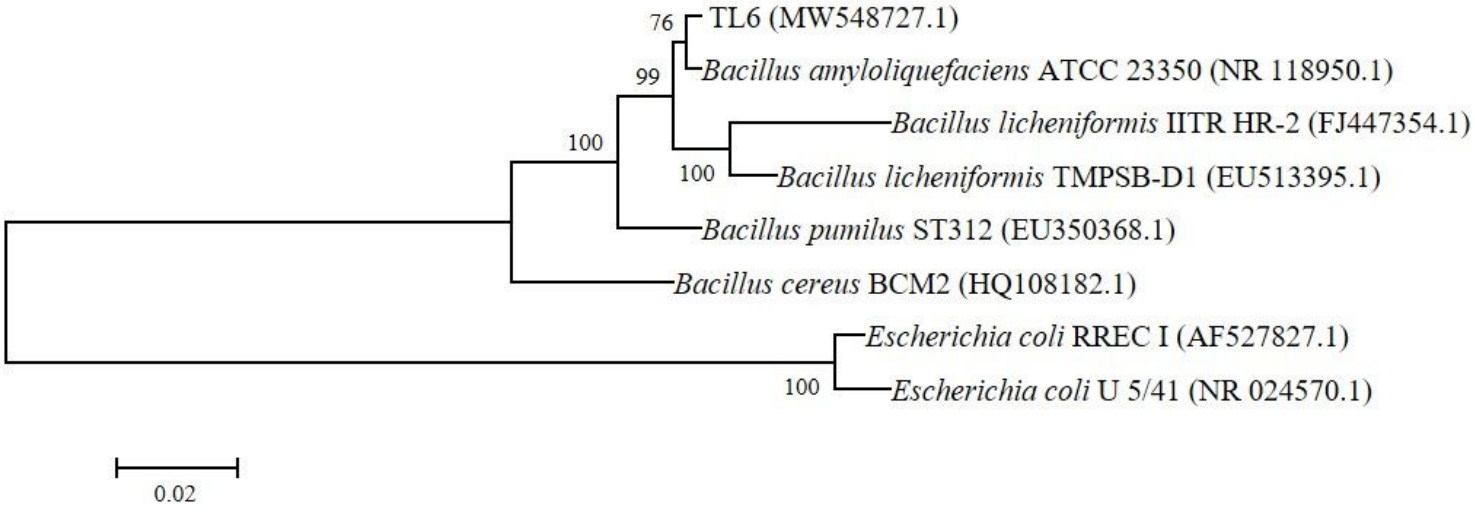

Figure 2

A phylogenetic neighbor-joining tree showing the relationship of the TL6 strain with other related species. Bootstrap values of 100 analyses are shown at the branch points. The scale bar represents two nucleotide substitutions per 100 nucleotides of 16S rDNA sequence.

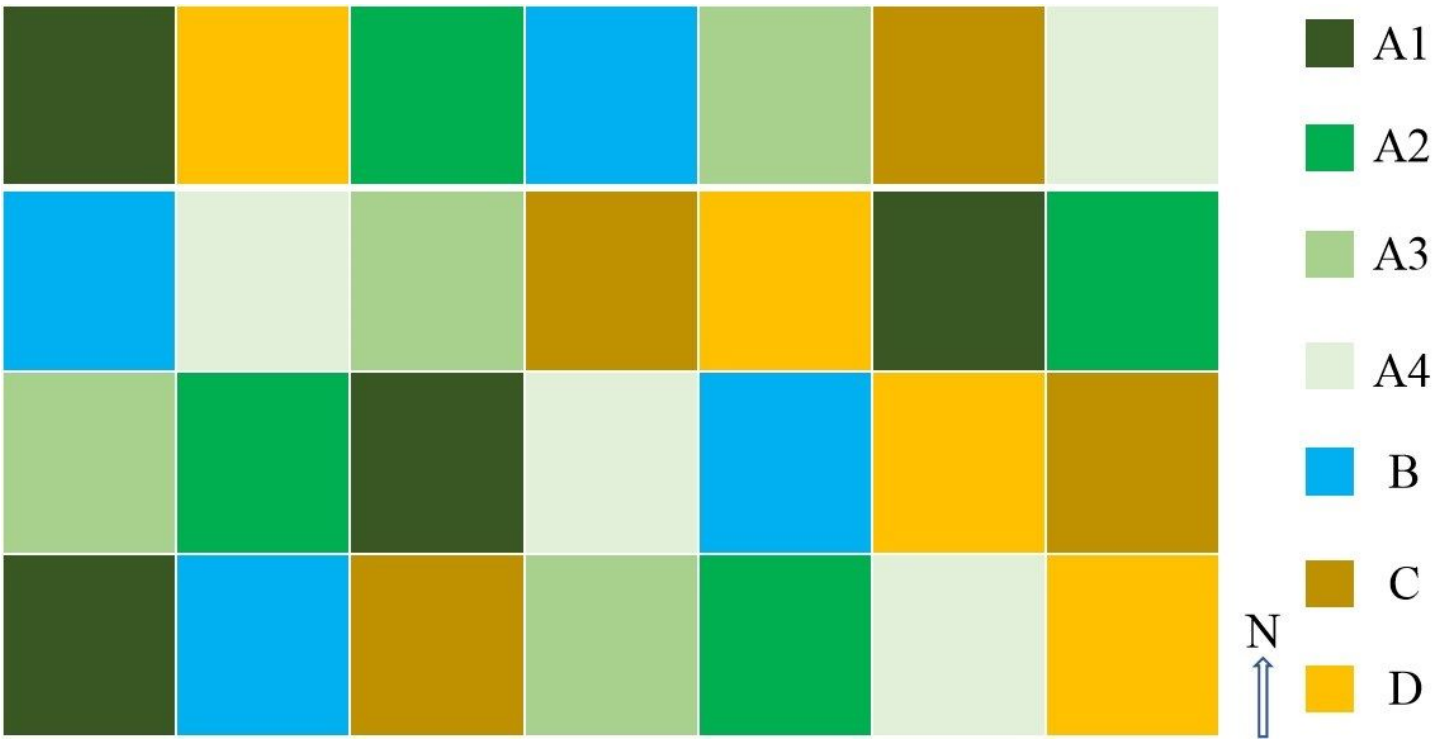

Figure 3

The layout of biological control efficiency in the field assay A1 represents the original fermentation solution of TL6 strain; A2 is A1 diluted 10x; A3 is A1 diluted 100x; A4 is A1 diluted 200x; B is in 1000 times of 500 g· L-1 Carbendazim suspension; C is A4+B; D is the control of clear water.