

ISOLATION AND CHARACTERIZATION OF BIOCONTROL BACTERIUM TL6 FOR PEANUT EARLY LEAF SPOT

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(Received 25th Oct 2022; accepted 20th Jan 2023)

Abstract. Peanut early leaf spot caused by *Passalora arachidicola* is serious fungal disease, which occurs in all production areas in China. Chemical control is the main method to prevent this disease. Nevertheless, it is easy to cause resistance, resurgence, residue problem (3R). Therefore, biological control is becoming the research hotspot for peanut early leaf spot control. 60 healthy peanut leaves were collected from major peanut production areas and were got for potential bacterial isolates. A total of 465 isolates were obtained. 22 strains showed differing levels of resistance against *P. arachidicola*. Among the antagonistic isolates, strain TL6 showed strongest inhibitory activity. It was identified as *Bacillus amyloliquefaciens* based on morphological characteristics, physiological and biochemical reactions as well as comparative analysis of its 16S rDNA sequence. The field control effect was above 69.17% after spraying the fermentation liquid of strain TL6. The field control effect of the strain TL6 fermentation liquid diluted 200 times and including the addition of 500 g/L carbendazim diluted 1000 times inhibited *P. arachidicola* by 81.33%. The combination of strain TL6 and carbendazim had a significant synergistic effect. Overall, strain TL6 showed promising potential commercial development and application in controlling peanut early leaf spot disease.

Keywords: *Passalora arachidicola*, biological control, inhibitory action, *Bacillus amyloliquefaciens*, control effect evaluation

Introduction

Peanut is an important economic and oil crop. China is the largest peanut producer in the world (Wang et al., 2022). Liaoning is a province with a large growing of peanut area. In recent years, the average annual area in Liaoning planted with peanuts has been approximately 370,000 hectares (Yu et al., 2018). 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 as well as lack of aflatoxins (Zhou et al., 2014a). 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 (Yu et al., 2019). 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% (Ghewande et al., 2001).

Currently, early leaf spot in peanut production is primarily controlled by chemical pesticides (Gangopadhyay et al., 1996; Nutsugah et al., 2007; Rakholiya et al., 2012). However, the problems of resistance, resurgence and residues caused by the long-term use of chemical pesticides have become increasingly prominent (Liu et al., 2010). Research

on disease management around the world primarily focuses on biological control or the joint management of biological agents and fungicides (Kumhar et al., 2018). During the past few decades, researchers had studied the antagonistic effects of a variety of fungal and bacterial biocontrol agents (Kishore et al., 2005; Couillerot et al., 2008). *Bacillus cereus* 304 significantly reduced the severity of early leaf spot on chitin-modified leaves (Kokalis-Burelle et al., 1992). It was clear 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 (Hasan et al., 2014). We isolated and screened microorganisms from peanut leaves to obtain strains with strong inhibitory activity against *P. arachidicola*. We would plan to verify the control effect of biocontrol agent against peanut early leaf spot with field experiments. The result would supply an effective prevention and control method and lay a theoretical foundation for the development of biocontrol agents against this peanut leaf disease.

Material and methods

Sample collection and bacterial isolation

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/min at 25 °C 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 °C 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 (Chen et al., 2005). 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 ($\Phi = 6$ 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 °C. Each treatment was repeated three times.

Antimicrobial spectrum assay

Bacterial isolate that had strong inhibitory activity namely strain TL6 was further selected to test against other fungal plant pathogens, including *Phytophthora capsica*, *Coniella diplodiella*, *Phytophthora infestans*, *Botrytis cinerea*, *Fusarium oxysporum*, *Colletotrichum orbiculare*, *Botryosphaeria berengeriana*, *Fusarium 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 plate that only cultured with the pathogen was served as the control. The plate was incubated at 25 °C. After 7 days of incubation, the ability of biocontrol agents to inhibit the pathogen was observed (Yoshida et al., 2001).

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 °C for 48 h. A 1 mL suspension of 1×10^9 CFU/mL 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 at 25 °C 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 peanuts were only cultivated and managed normally, i.e., they were fertilized and irrigated normally, but no other chemicals were sprayed on the plants. 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), with a total of seven treatments (Zhou et al., 2014b). The treatments included the original fermentation solution of strain TL6, 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 1*).

Table 1. The experimental treatment (n = 4)

Letter	Treatment	Letter	Treatment
A1	Original fermentation solution of TL6	B	50% Carbendazim suspension diluted 1000 times
A2	Original fermentation solution of TL6 diluted 10 times	C	Original fermentation solution of TL6 strain diluted 200 times + 50% 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		

Each treatment had 30 holes of peanuts. The experimental plots were randomly arranged (*Fig. 1*). 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 (GB/T 17980.85-2004). The leaf grading method was divided into six 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.

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

$$I(\%) = \frac{Z_{ck} - Z_x}{Z_{ck}} \times 100$$

where A = Number of diseased leaves of all levels; B = the level of each diseased leaf; M = total number of leaves; B_{max} = the highest level of disease; I = control effect; Z_{ck} = the disease index of control group, and Z_x = the disease index of treatment group.

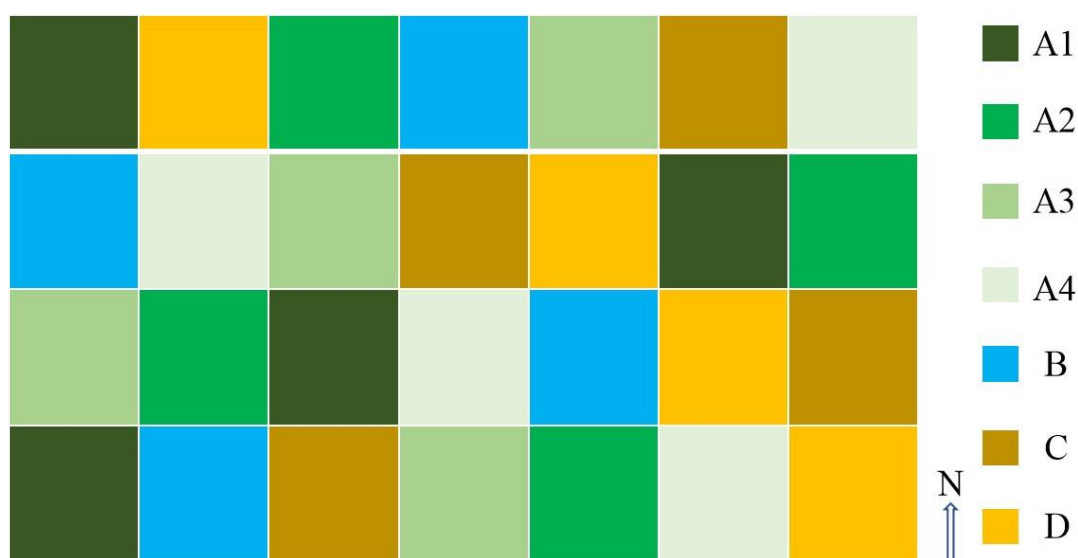


Figure 1. The layout of biological control efficiency in the field assay. A1 represents the original fermentation solution of TL6 strain; A2 is A1 diluted 10x; A3 is A1 diluted 100x; A4 is A1 diluted 200x; B is in 1000 times of 50% Carbendazim suspension; C is A4 + B; D is the control with clear water

Identification of strain TL6

Physiological and biochemical tests

The physiological and biochemical tests were conducted (Dong et al., 2001). 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.

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/mL of lysozyme). The chromosomal DNA was extracted (Zhang et al., 2016).

The 16S rDNA was amplified by PCR with the universal primers 27f (5'-AGAGTTTGGATCCTGGCTCAG-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 ddH₂O. The amplification conditions were established for the initial denaturation at 95 °C (5 min), followed by 35 cycles at 95 °C (30 s), 57 °C (45 s) and 72 °C (1 min 30 s), with a final extension at 72 °C for 5 min. The PCR products were sequenced by Shanghai Sangon Biotech, China (<https://www.sangon.com/>). The sequences were clustering analysis using the Mega program (version MEGA7.0) 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).

Results

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

A total of 465 bacterial isolates was obtained from peanut leaves. The results showed that about 4.73% (22 out of 465 isolates) showed differing degrees of inhibition against *P. arachidicola* (Table 2). Among them, strain TL6 showed the strongest inhibitory effect on *P. arachidicola*, and the diameter of inhibition zone was 64.3 mm (Fig. 2).

Table 2. The inhibitory effect of different isolates against *P. arachidicola* ($n = 3$)

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

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

Antimicrobial spectrum assay of strain TL6

Strain TL6 had differing degrees of inhibition on 10 types of plant pathogens, and the inhibitory zones were ranged from 6.2-20.3 mm. The inhibitory activity of strain TL6 was found highest against *P. arachidicola* (20.3 ± 0.45 mm), and the lowest against *B. berengeriana* (6.2 ± 0.62 mm) (Table 3).

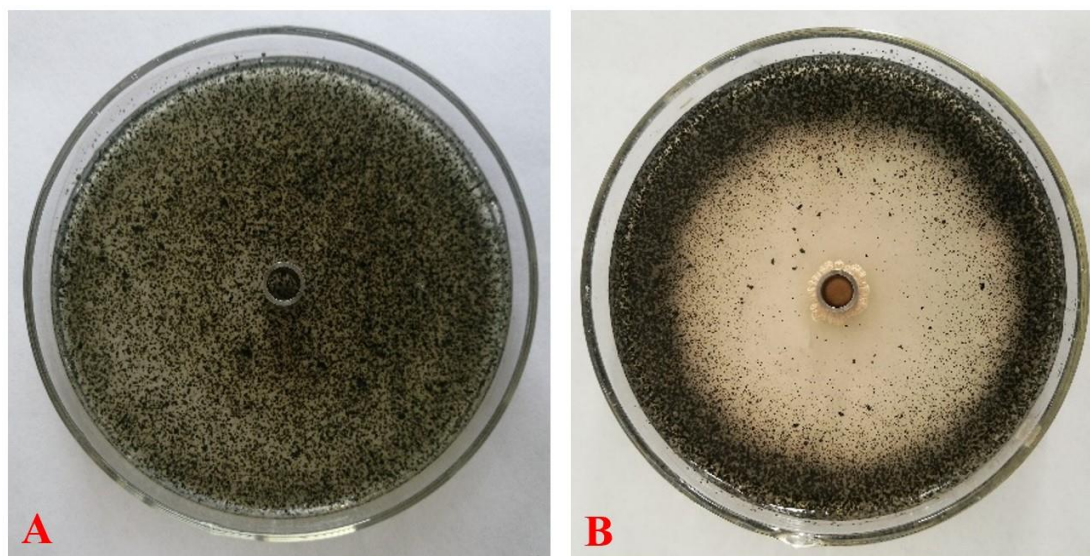


Figure 2. The efficacy of TL6 strain against *C. arachidicola* in PDA plates. A: control; B: treatment with strain TL6

Table 3. Inhibitory spectrum of strain TL6 against pathogens (n = 3)

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

Means within the same column followed by different letters (a, b, c, d, e, f, g) 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 strain 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/L carbendazim suspension diluted 1000 times. The control effect of the original fermentation solution of strain 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 strain TL6 fermentation solution diluted 200 times + 500 g/L 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 4).

Identification 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 °C. 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 5).

Table 4. The control effect of strain TL6 against *Cercospora arachidicola* in the field ($n = 4$)

Treatment	2019		2020	
	Average disease index	Control effect (%)	Average disease index	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	-

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

Table 5. The physiological and biochemical characteristics of strain TL6 ($n = 3$)

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

+ indicates a positive reaction. - indicates a negative reaction

The sequencing results showed that the 16S rDNA of strain TL6 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 (Fig. 3). Based on its physiological and biochemical characteristics and 16S rDNA gene sequence, strain TL6 was putatively identified as *B. amyloliquefaciens*. The 16S rDNA sequence of strain TL6 was deposited in the GenBank (MW548727.1).

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 (Dai et al., 2016). Extensive research on the development and utilization of *Bacillus* for biological control have been conducted in China and throughout the world Zou et al., 2017). 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, strain TL6 isolated from peanut leaves that putatively related to *B. amyloliquefaciens* was found 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 strain 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 strain TL6 fermentation liquor was relatively stable.

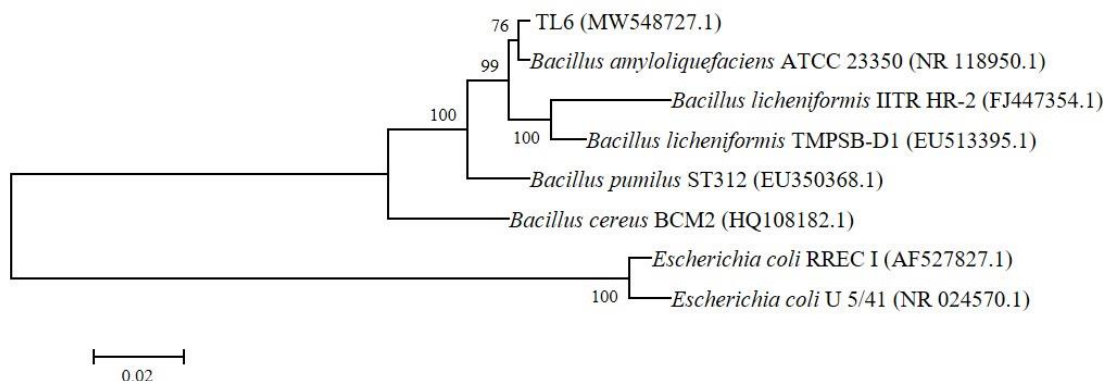


Figure 3. A phylogenetic neighbor-joining tree showing the relationship of the TL6 strain with other related species. Bootstrap values of 100 analyses are shown at the branch points. The scale bar represents two nucleotide substitutions per 100 nucleotides of 16S rDNA sequence

Bacillus amyloliquefaciens was named by Fukomato in 1943 and was not included on the Approved Lists of Bacterial Names and had not been validly published since January 1, 1980 (Priest et al., 1986). *B. amyloliquefaciens* was 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 (Wang et al., 2017; Almaghrabi et al., 2013). *B. amyloliquefaciens* had a broad spectrum of inhibitory activity that contains rich and diverse active substances, primarily including antagonistic proteins (Qin et al., 2015), surfactins (Mikkola et al., 2004), iturins (Hiradate et al., 2002), and chitinase (Yan et al., 2018). These active substances played 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* (He et al., 2015). *Bacillus amyloliquefaciens* B190 was effective at controlling lily grey mold (*Botrytis elliptica*), and the inhibitory effect was even significantly higher than the commonly used fungicides prochloraz and acetamioxime (Chiou and Wu, 2003).

As a potential biocontrol microbial resource, *B. amyloliquefaciens* had not been used to control peanut early leaf spot. In this study, we found that strain 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 an important security measure 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.

Conclusion

The results of this study demonstrated that *Bacillus amyloliquefaciens* TL6 could be applied in peanut of economic interest for biological control against peanut early leaf spot caused by *Passalora arachidicola*. The biocontrol agent showed promise for commercial development and application in controlling peanut early leaf spot disease, and could serve as agents for the long-term prevention and control of this important disease, and simultaneously this biocontrol agent could reduce of chemical pesticides in peanut production.

Acknowledgements. We appreciate very much to Dr. Shuyi Yu from Institute of Plant Protection, Liaoning Academy of Agricultural Sciences and Dr. Tianya Li from College of Plant Protection, Shenyang Agricultural University for critical reading of our manuscript.

Funding. Liaoning Province Youth Top Talent Project (XLYC1907181), Basic research expenses of Liaoning Academy of Agricultural Sciences (2021GQ1903), National Key Research and Development Program of China (2017YFD0201100), Basic Research Expenses of Liaoning Academy of Agricultural Sciences (2022XTCX0502003).

Competing interests. The authors declare that they have no competing interests.

Availability of data and materials. The datasets used and analyzed during this study are available from the corresponding author upon reasonable request.

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