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RESEARCH ARTICLE

Screening and Identification of the antagonistic strain DL-59 of *B. velezensis* against *A. brassicae* and biocontrol efficiency

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Abstract **The aim of this study was to screen the antagonistic spore-forming bacteria of *Alternaria brassicae* and to assess their control effect. The test microorganism was *A. brassicae* in this experiment. One spore-forming strain named DL-59 with a rather stronger antagonistic activity than ever reported was obtained by using an improved agar plate diffusion method. Morphological, physiological and biochemical tests combined with 16S rDNA sequence analysis were carried out to identify DL-59. Its control effect was studied in the basin culture and the plot trials. Two hundred strains were isolated form soil and 20 antagonistic bacteria strains were obtained through preliminary screen. After the secondary screening, a strain named DL-59 with a rather stronger antagonistic activity was obtained and identified as *B. velezensis* with the largest diameter of inhibition zone of 23 mm. The control efficacy was 79.07%. The plot trials showed that DL-59 had a visible preventive effect on cabbage black spot disease. This can be applied for biological control of plant disease. The strain can produce spores, which is important for the storage of its preparation.**

Keywords antagonistic bacterium, screening and identification, 16S rDNA, control effect

Introduction

Black leaf spot disease of cabbage is also called black spot disease and it is a widespread fungal disease with a great negative effect on the yield of Chinese cabbage. Nearly 90% of the *Alternarias* reported and described in the world are facultative parasites in different species of plants. They can cause a variety of leaf spot diseases (Cui et al., 2005). Among them, *Alternaria brassicae*, *A. brassicicola* and *A. japonica* can cause black spot disease of cruciferous vegetables and infect many cruciferous vegetables through weeds, plant residues and seeds of *Brassica* and *Raphanus*. Different methods were used to prevent or control cabbage spot disease, such as selection of disease-resistant varieties, seed treatment, strengthening the field management, chemical control, biological control and so on.

Generally, biological control, as a promising control method, does not adversely affect other beneficial microorganisms in the agroecosystems. At the same time, with the properties of high biocide activity and low dosage, it is easy to be decomposed by edaphon, avoiding accumulation in nature. When the antagonistic strain is used for biological control, it should be easy to be implemented in large-scale industrial production with microbial agents having high activity, strong stability, and long shelf life. Therefore, this experiment is committed to screen the spore-producing antagonistic bacteria due to easy control of the liquid fermentation of bacteria and the spores resistance to heat, pressure and chemical agent. These make the commercialization of the microbial agents more realistic.

A. brassicae was used as test microorganism. After the preliminary screening and the secondary screening, Strain DL-59 with a relatively high rivalry activity was obtained. The morphological, physiological and biochemical characteristics and the 16S rDNA sequence of this strain were further studied. Finally, it was identified as *B. velezensis*. Through its identification, more details of its physiological and biochemical characteristics were obtained which laid a good foundation for the studies on the mechanism of bacterial antagonism. The basin culture and the plot trials were also

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carried out for preliminary study on the prevention of cabbage black spot disease.

Materials and methods

Materials

The tested pathogen was *A. brassicae* preserved in the bio-pharmaceutical laboratory of College of Life Science, Agricultural University of Hebei, China.

NA medium, NB medium and potato medium (PDA) were prepared in this study according to the "Microbiology Experiment" (Shen et al., 1999), while the media for identifying physiological or biochemical characteristics were prepared according to the "Manual of System Determinative Common Bacteriology" (Dong and Cai, 2001). The fermentation medium for the secondary screening contained 3.0% corn meal, 3.0% beef extract, 0.05% K_2HPO_4, and 0.05% $CaCl_2$ at the initial pH of 7.0.

Reagents for identifying physiological or biochemical characteristics of DL-59 were used in this study according to the "Manual of System Determinative Common Bacteriology" (Dong and Cai, 2001). Reagents for 16S rDNA sequence analysis were used following the "Short Protocols in Molecular Biology 4th Edition" (Ausubel et al., 1999).

Test methods

Soils at the depth of 10–15 cm were collected by shovel from diseased cabbage fields, and then stored in plastic bags after airdrying, with sampling time and locations recorded.

Isolation and purification of bacteria from soils were conducted by the following procedures. 1.0 g soil sample was ground and added into tubes with 9.0 mL sterile distilled water, then shaken for 20 min in water bath at 80°C. Next, 1.0 mL sample solution was diluted into 1×10^{-6} by ten times gradient dilution, and then 0.1 mL diluent was coated on the plate of NA medium in two gradient concentrations of 1×10^{-4} and 1×10^{-5}, respectively, cultured and inverted for 24 h at 30°C. Single colonies in different shape, size and color were transferred respectively into the slant of NA medium. Then, the strains numbered and cultured for 24 h at 30°C were finally stored in the refrigerator at 4°C for future use, and the purification of each strain was conducted and tested by Streak Plate Method.

To activate the strains, they were transferred into the slant of NA and PDA medium respectively after the cabbage black spot pathogen and the isolated bacteria from 4°C fridge were kept at room temperature for 1 h. The isolated bacteria were cultured at 30°C for 48 h and the cabbage black spot pathogen was stored at 25°C for 7–10 d for future use.

Preliminary screening for the antagonistic bacteria against *A. brassicae* was conducted as follows. 5.0 mL sterile distilled water was added into the slant of *A. brassicae* cultured for 10 d. Spores were scraped slightly with inoculation loop and shaken up to obtain the spore suspension,which was added into the PDA medium, cooled to 50°C after melting by shaking up rapidly. Thereafter, the spore suspension was poured on the plate and prepared for future use after solidification. Some thalli were selected from the slant of the isolated bacteria with inoculation loop and inoculated on the PDA medium plate to culture for 24–48 h at 30°C. Finally, antagonistic strains with the inhibition zone were selected for future use.

The secondary screening for the antagonistic bacteria against *A. brassicae* was done by inoculating the antagonistic bacteria obtained from the preliminary screening into the 250 mL erlenmeyer flask with 50 mL activated fermentation medium, which was shaken in rotary vibrator for 48 h at 30°C at 180 r/min. Sterile membrane filter (pore size of membrane filter is 0.22 μm) was used to remove the thalli. 5.0 mL sterile distilled water was added into the slant of *A. brassicae* cultured for 10 d with spores scraped slightly with inoculation loop and shaken up to obtain the spore suspension. Then, the spore suspension was added into the PDA medium and cooled to 50°C after melting by shaking up rapidly. Finally, the spore suspension was poured on the plate and prepared for future use after solidification.

Regular pores were perforated with the puncher after the medium was cooled. 70 μL antagonistic bacteria broth fermented already for 48 h without the thalli was added into each pore and cultivated for 3 d at 30°C after standing for 30 min. Finally, the diameter of inhibition zone was measured and the strains with large and clear inhibition zone were selected.

Methods of bacteria identification

Morphological identification

To observe the colonial morphology, some thalli were selected from the slant of the isolated bacteria with inoculation loop, the thalli were put into a tube with 10.0 mL sterile distilled water, and then the tube was shaken. 1 mL was taken into another tube with 9 mL sterile distilled water, it was diluted into a variety of concentrations of bacterial suspension. 0.1 mL diluents with two gradient concentration of 10^{-4} and 10^{-5} were coated on the plate of NA medium respectively and cultured invertly for 24 h at 30°C.

Some thallus of the isolated bacteria were selected from the slant for gram stain or spore staining, and the staining method was described in the " Manual of System Determinative Common Bacteriology " (Dong and Cai, 2001). The morphological characteristics of thalli and spores were observed by the microscope.

Physiological and biochemical identification

According to the "Manual of System Determinative Common

Bacteriology" (Dong and Cai, 2001) the antagonistic bacteria were identified by many physiological biochemical tests (Table 2) such as nitrate reduction, indole test, hydrogen sulfide, starch hydrolysis, and citrate test.

Extraction of the genomic DNA and PCR reaction

The genomic DNA of the antagonistic bacteria was extracted (Sambong et al., 1995; Rainey et al., 1996). The quality of DNA was detected by 1% agarose electrophoresis. The concentration of the DNA was measured by the BIO-RAD QuantityOne software. Primers are the universal primer (Lane, 1991), forward primer is 27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer is 1495R: 5′-CTACGGCTACCTTGTTACGA-3′. They are respectively located at 8–27 bp and 1495–1514 bp of 16S rDNA (the *Escherichia coli* as a standard). The PCR reaction system was as follows: DNA (70 ng/μL) template 2 μL; dNTP mixture (2.5 mmol/L) 3.5 μL; 27F (20 μmol/L) 1.0 μL; 1495R (20 μmol/L) 1.0 μL; 10 × ExTaq buffer (Mg^{2+} pluse) 5 μL; ExTaq DNA polymerase 0.5 μL; and ddH_2O 50 μL. The PCR amplification conditions were as follows: denature at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, annealing at 55°C for 1 min and at 72°C for 3 min, with the final extension at 72°C for 5 min. After purification, the PCR products were sent for sequencing (Sagon Company).

16S rDNA sequence analysis and phylogenetic tree construction

Resemblance analysis of the 16 S rDNA sequence was done through the GenBank database using the BLAST method. Multiple alignments were carried out among the sequences with high resemblance in the Clustal X (1.8) program. Finally, a multiple alignment array was established, with gaps instead of lines, and a phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987).

The basin culture trials

The field soil was taken, sterilized, and then put into the nutrition pots. One healthy cabbage seedling was transplanted into each nutrition pot, cultured and managed normally. The spores of *A. brassicae* cultivated for 7 days were washed with 0.1% Tween 80. The concentration of spores was adjusted to make sure that there were 10 spores in per field of microscope (10 times) on average. The spore suspension was evenly applied on the cabbage leaves using a writing brush. After 60 h, the fermented liquid of DL-59 (at about 10^9/mL) was diluted to 5 times volume. These cabbages were treated in three groups with the diluted fermentation liquor as Group 1, Group 2 treated with the 50% carbendazol wettable powder that had been diluted to 500 times as medicine control, and Group 3 treated with the water as a blank control. There were 20 cabbage seedlings in each group, with 3 replicates. In 6 d, the cabbage seedlings were removed to investigate the incidence of cabbage black spot disease (Table 1) and calculate the disease index and the control efficacy by the flowing equation:

Table 1 Cabbage black spot disease classification standard

Disease level	Degree	Numeric value
1	No symptoms	0
2	Leaves with small brown spots, l–3	1
3	Leaves with brown spots, 4–8	2
4	Leaves with brown spots, 9 or more	3

$$I = (N \times R)/(M \times T),$$

where I is disease index, N is the number of the diseased plant in a certain disease level per plant, R is the numeric value of the corresponding disease level, M is the highest numeric value of the investigated cabbage, and T is the total number of cabbage investigated per plant.

Control efficacy can be calculated with the following equation (Zhang, 2001):

Control efficacy (%) = [(CK Disease index − Pt Disease index)/CK Disease index] × 100.

The field with Chinese cabbages planted for 2 years was selected at the Agricultural University of Hebei. The spore suspension of *A. brassicae* was sprayed on young seedling of these cabbages. 60 h later, these cabbages were treated twice in a row with the fermented liquid of DL-59 (concentration was about 10^9CFU/mL) diluted to 5 times volume as Group 1, treated with the 50% carbendazol wettable powder diluted to 500 times as Group 2 (a medicine control), and treated with the water as Group 3 (a blank control). There were 20 m^2 in each group, with 3 replicates. The incidence of cabbage black spot disease was investigated. The disease index and the control efficacy were calculated.

Results

Preliminary screening for the antagonistic bacteria

More than two hundred strains were isolated from soil samples, 20 strains with larger diameter of inhibition zone and stronger antibacterial activity than the other strains were obtained through preliminary screening. Fig. 1 shows the antagonistic effect of isolated bacteria strains against the pathogen by preliminary screening.

Secondary screening for the antagonistic bacteria

Twelve strains with better antagonistic activity than the other 8 strains were obtained from the secondary screening. The diameter of inhibition zone for 6 strains were more than 12 mm, the growth of cabbage black spot pathogen was effectively inhibited. The diameter of inhibition zone for strain DL-59 reached 23 mm, and strain DL-59 had the

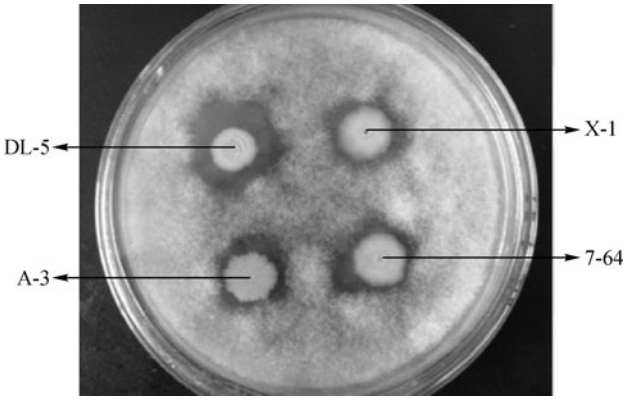

Figure 1 Antagonistic effect of isolated bacteria strains against the pathogen.

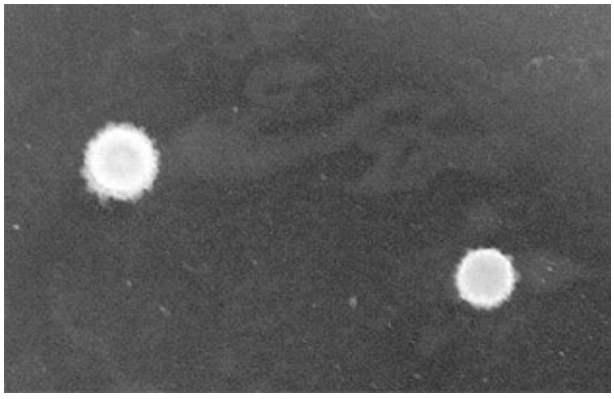

Figure 3 Colony morphology of DL-59 on NA.

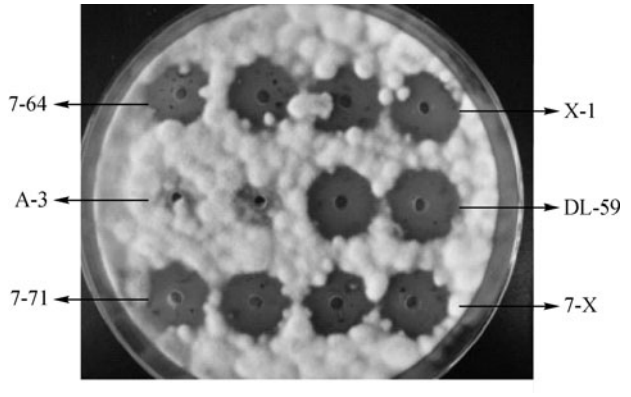

Figure 2 Antagonistic effect of isolated bacteria strains against the pathogen by the secondary screening.

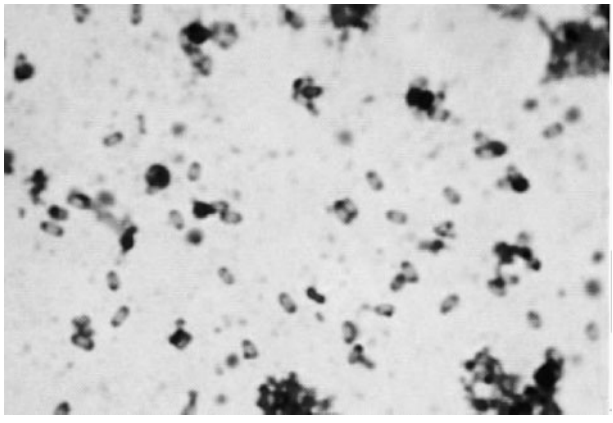

Figure 4 Morphological shapes of thallus and spore of the antagonistic bacteria strain DL-59.

highest antagonistic effect on *A. brassicae* among the strains investigated. Figure 2 shows the antagonistic effect of isolated bacteria strains against the pathogen by secondary screening.

The identification of antagonistic bacteria

Colonial morphology of the strain DL-59

The single colony of the antagonistic strain DL-59 on NA medium plate was circular, yellowish and opaque with irregular edges, and uneven and wrinkly surface. Furthermore, there was depression in the middle, showing cavernous in shape (Fig. 3).

Thallus morphology of the strain DL-59

Cells of strain DL-59 were rod-shaped under the microscope after staining at about 2.1 μm length and 0.6 μm width while the Gram stain showed positive. The thallus formed ellipsoidal and swelled spores, and then budded from the middle (Fig. 4). The test of motility and metachrome showed positive while the acid-fast stain showed negative.

The results of physiological and biochemical identification

Physiological and biochemical identification of the strain DL-59 was shown in Table 2. Strain DL-59 was identified as *Bacillus* (Dong and Cai, 2001; Buchanan and Gibbons, 1984).

16S rDNA complete sequence analysis of strain DL-59

The sequence length of 16S rDNA of the strain DL-59 is 1460. The resemblance analysis was carried out between this sequence and other bacterial 16S rDNA sequences using the BLAST program. Strain DL-59 and the other nine strains together formed a cladogram according to resemblance. Based on the results shown in Fig. 5 and Table 3, the DL-59 strain was preliminarily identified as *Bacillus*. Furthermore, the similarity between the DL-59 strain and the strain AY603658 and AB245422 of *B. velezensis* was 99.78%. According to its morphological characteristics, physiological and biochemical characteristics and 16S rDNA sequences analysis, the strain DL-59 was identified as *Bacillus velezensis*.

Table 2 The results of physiological and biochemical identification

Item	Result	Item	Result
Growth temperature and heat resistance 65°C	+	Sugar and spirits fermentation	+
Growth temperature and heat resistance 45°C	+	Methyl red	−
Growth temperature and heat resistance 37°C	+	V.P determination	−
Growth temperature and heat resistance 4°C	−	Starch hydrolysis	+
Nitrogen sources utilization$(NH_4)_2HPO_4$	+	Production of dextrine crystallization	−
Nitrogen sources utilization KNO_3	−	Cellulose decomposition	+
Carbon sources utilization mannose	+	3 − ketone group lactose	+
Carbon sources utilization maltose	+	NNitrate reduction	−
Carbon sources utilization sucrose	−	Nitrite reduction	+
Carbon sources utilization soluble starch	+	Denitrification	−
Fluorochrome	−	Ammonia production	+
Production of pyocyanin	−	Urease	−
Malonic acid utilization	−	Phenylalanine deaminase	+
Citrate utilization	−	Tryptophan deaminase	+
Tartrate utilization	−	Lipase test	+
Catalase	+	Hydrogen sulfide	+
Oxidation and ferment of glucose	fermentative	Milk decomposition	peptonization
Indole	+		

"+" means positive while "−" means negative.

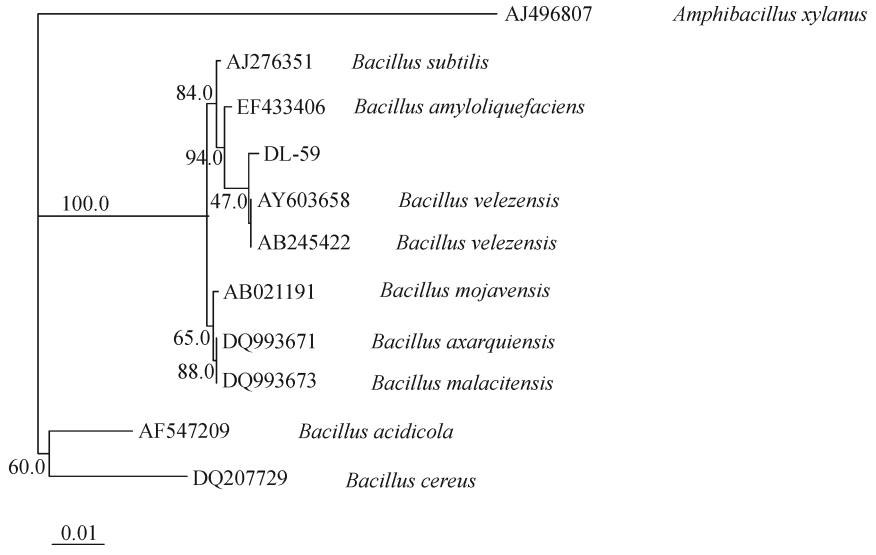

Figure 5 Phylogenetic tree of 16S rDNA sequence of DL-59 strain and related strains.

Table 3 Comparison of similarity between antagonistic strain DL-59 and several norm stains

Sequence number	Species	Strain-number	Similarity (%)
AJ496807	*Amphibacillus xylanus*	DSM 6626	87.90%
AJ276351	*Bacillus subtilis*	DSM10	99.42%
EF433406	*Bacillus amyloliquefaciens*	BCRC 11601	99.57%
AY603658	*Bacillus velezensis*	CR-502	99.78%
AB245422	*Bacillus velezensis*	LMG22478	99.78%
AB021191	*Bacillus mojavensis*	IFO15718	99.28%
DQ993671	*Bacillus axarquiensis*	LMG 22476	99.28%
DQ993673	*Bacillus malacitensis*	LMG 22477	99.28%
AF547209	*Bacillus acidicola*	105-2	93.87%
DQ207729	*Bacillus cereus*	CCM 2010	93.19%

Control effect

The result of basin culture trials

The result of basin culture trials (Table 4) showed that, compared with the blank control, the treatment with DL-59 significantly reduced the disease index, with 76.37% control efficacy, indicating a higher certain preventive effect on cabbage black spot disease than that of 50% carbendazol wettable powder diluted to 500 times.

The result of the plot trials

The result of the plot trials (Table 5) showed that DL-59 had a highly visible preventive effect on cabbage black spot disease, with 79.07% control efficacy higher than the 50% carbendazol wettable powder diluted to 500 times.

From the growth condition of cabbages, plants treated by DL-59 grew more vigorously. The strain DL-59 would secrete certain materials prompting plant growth. The mechanism needs further research. Fig. 6 shows the diseased plant after artificial inoculation of the *A. brassicae*. Fig. 7 shows the healthy plant treated with the fermented liquid of DL-59 after artificial inoculation of *A. brassicae*.

Discussion

The safety in vegetable production has attracted wide public concerns all over the world, the application of biological control microbes and their secondary metabolites, antibacterial activity of plant material, phytoalexin, anti-fungal protein and the efficient, broad-spectrum, lower toxic, less-persistent chemicals, as widely concerned hot issues, are virtually worth

Table 4 The basin culture trials control effect of DL – 59 against cabbage black spot disease

Treatment	Treatment pattern	$P = 0.05$	Total number	Diseased plant	Disease index	Control efficacy (%)
DL – 59	spray	a	60	13	16.85	76.37
Carbendazol wettable powder	spray	a	60	15	17.82	75.00
Water	spray	b	60	55	71.30	–

"–" means no control effect.

Table 5 The plot trials control effect of DL – 59 against cabbage black spot disease

Treatment	Treatment pattern	$P = 0.05$	Total number	Diseased plant	Disease index	Control efficacy (%)
DL – 59	spray	a	120	9	7.50	79.07
Carbendazol wettable powder	spray	a	120	14	28.13	78.51
Water	spray	b	120	43	35.83	–

"–" means no control effect.

Figure 6 Diseased plant after artificial inoculation of *A. brassicae.*

Figure 7 The healthy plant sprayed with the fermented liquid of DL-59 after artificial inoculation of *A. brassicae.*

investigating. The *bacillus* is the predominant microorganism in the microecology of soil and plants because it can produce endogenous spore, with very strong biotic potential,for industrial production, disease resistance and stress tolerance. Furthermore, it is a nonpathogenic bacterium widely existing in the nature, harmless to humans and animals without polluting the environment (Chen et al., 2003). Many natural isolated strains with excellent traits have been successfully applied to biological control of plant diseases (Chen et al., 2003).

There were some reports on antagonistic bacteria for biological control against cabbage black spot disease. SivaPalan determined and confirmed that the antagonistic microorganisms isolated from different batches of broccoli seeds had an inhibition effect on the spore germination of the *A. brassicicola* in vitro conditions (Sivapalan, 1993). Leifert isolated the antagonistic microorganisms from a plant of *Brassica*, and the result of the leaf disc cocultivation showed that the isolates from *Pseudomonads fluoescens*-CL42, 66 and 82, Serratia plymuthica-CL43 and *S. liquefaciens*-CL80 all can effectively inhibit the growth of *A. brassicae* (Leifert et al., 1992). Among the antagonistic microorganisms isolated from the leaves of kale, the isolate form BR-11 (*Bacillus* spp.) and BS-25 (*Bacillus* spp.) can effectively inhibit the growth and the spore germination of *A. brassicicola* (Chung and Huang, 1994).

In this study, an antagonistic bacterium named DL-59 was identified as *Bacillus velezensis* with a strong antibacterial activity and 79.07% control efficacy. It has a high application value in plant disease for biological control. Therefore, further research should be carried out, such as the research on bacteriostatic mechanism, the fermentation condition optimization, using genetic engineering methods to build more efficient biological control strains. In all, it demonstrated that DL-59 is a nice biocide strain for controlling cabbage black spot disease, a wide-spread disease around vegetables. The further investigation would be concentrated on the practice of large-scale industrial production, such as the cost and fermentation conditions, etc.

Acknowledgements

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