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Isolation, identification, and biocontrol of antagonistic bacterium against *Botrytis cinerea* after tomato harvest

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ABSTRACT

Tomato is one of the most important vegetables in the world. Decay after harvest is a major issue in the development of tomato industry. Currently, the most effective method for controlling decay after harvest is storage of tomato at low temperature combined with usage of chemical bactericide; however, long-term usage of chemical bactericide not only causes pathogen resistance but also is harmful for human health and environment. Biocontrol method for the management of disease after tomato harvest has great practical significance. In this study, antagonistic bacterium B-6-1 strain was isolated from the surface of tomato and identified as *Enterobacter cowanii* based on morphological characteristics and physiological and biochemical features combined with sequence analysis of 16SrDNA and *ropB* gene and construction of dendrogram. Effects of different concentrations of antagonistic bacterium *E. cowanii* suspension on antifungal activity after tomato harvest were analyzed by mycelium growth rate method. Results revealed that antifungal activity was also enhanced with increasing concentrations of antagonistic bacterium; inhibitory rates of 1×10^5 colony-forming units (cfu)/mL antagonistic bacterial solution on *Fusarium verticillioides*, *Alternaria tenuissima*, *and Botrytis cinerea* were 46.31%, 67.48%, and 75.67%, respectively. By using *in vivo* inoculation method, it was further confirmed that antagonistic bacterium could effectively inhibit the occurrence of *B. cinerae* after tomato harvest, biocontrol effect of 1×10^9 cfu/mL zymotic fluid reached up to 95.24%, and antagonistic bacterium *E. cowanii* has biocontrol potential against *B. cinerea* after harvest of fruits and vegetables.

Introduction

According to the database of Food and Agriculture Organization of the United Nations, tomato yield, an important economic product, in China ranks first in the world. However, tomato decay after harvest has been a major issue in the development of tomato industry. Currently, the control of tomato diseases after harvest is mainly through prevention and cure using chemicals. Nevertheless, long-term

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use of chemicides leads to many drawbacks such as environmental pollution, effects on human health, and drug resistance. With increasing awareness on food safety, restriction on the use of chemicides is also increasing.[1] Biological control methods have been developed in laboratories and commercially applied.[2] A study has demonstrated that antagonistic microbes to control diseases is a new alternative with great potential. In the biocontrol technique for preventing pathogenic decay after the harvest of fruits and vegetables, the main action is through antagonistic interaction among microbes, by changing the microbiological environment on fruit surface to promote reproduction of antagonistic bacteria and inhibiting the growth of pathogenic microorganisms, consequently reducing decay. Due to its excellent biocontrol effect, no harm to humans or animals, no environmental pollution or residue, and maintenance of good quality agricultural products, this method is considered to be one of the most important methods for replacing chemicides.[3–5] Till date, a number of antagonistic microorganisms have been selected from >10 types of fruits such as orange, apple, peach, pear, kiwifruit, and fresh jujube, and biocontrol organisms isolated are mainly small filamentous fungi,[6] bacteria,[7,8] yeast,[9,10] *etc*. At present, some companies outside China have successfully selected antibacterial agents, and some of them have been developed as products for commercial use such as American "Aspire" (Candida oleophila strain-182), "Biosave-100", "Biosave-110," and "Yield Plus" (*Cryptococcus albidus*), *etc*.

Researchers have found that the main pathogens causing the decay after harvest were botrytis caused by *Botrytis cinerea*, alternaria caused by *Alternaria tenuissima*, and fruit decay caused by *Fusarium moniliforme*. Because *B. cinerea* can tolerate low temperature, produce large spore yield, and has short period of onset of fruit diseases, botrytis is the most severe disease after tomato harvest, and hence prevention and cure of botrytis is a key approach for the protection and development of tomato products worldwide.[11] Recently, there are some reports[12–14] regarding biocontrol against *B. cinerea* of tomato. It has been reported that *Cryptococcus laurentii* effectively reduced *B. cinerea* in post-harvest tomato and post-harvest decay caused by *Pythium*[12]; Wang et al. found that inoculation of 1×10^8 cfu/mL cell suspension of sea yeast *Rhodosporidium paludigenum* reduced disease rate of tomato by 42.1%, when compared with control group 5 days after inoculation at 25 °C. Duan et al.,[13] showed that inoculation of suspension of 1×10^6 cfu/mL *Paenibacillus peoriae* BC-39 can effectively control *B. cinerea* in post-harvest tomato; 5 days after inoculation at 25 °C, the rate of protective effect against decay was 88.4% and the rate of therapeutic effect against decay was 79.9%. Moreover, rate of weight loss was significantly lower than the control after tomatoes were soaked in the prepared suspension.[14] Although some effective bacterial biocontrol agents have been reported, the number of identified biocontrol bacteria after harvest is evidently insufficient compared with other fields of biocontrol measures. In this study, botrytis was used as a target agent after harvest of tomatoes, and the selected B-6-1 strain with antibacterial activity was successfully isolated. B-6-1 strain was identified according to the morphological characteristics, physiological and biological features, and by sequence analysis of *16S rDNA* and *ropB* gene and dendrogram construction methods. The biocontrol effect of antagonistic bacterial strain after the harvest of tomato was explored to provide evidence for the prevention of botrytis in tomato and to develop research on biocontrol agents of tomato.

Materials and methods

Materials

The seeds of tomato (*Lycopersicon esculentum* Mill., hezuo909) were from Changzheng tomato seed testing ground at Shanghai. The seeds were picked up from Xiaodian district, Taiyuan city in Shanxi Province and were used for isolation, inoculation, and preservative experiments. Cherry tomato seeds (*L. esculentum* var. *cerasiforme*, Hongshengnv) were from KeFeng Seed Industry Co., Ltd (Changchun city of Jilin Province) and were picked up from Xiaodian district, Taiyuan city in Shanxi Province for the selection of antagonistic bacteria.

Antagonistic bacteria were isolated from the surface of tomatoes and purified. Tomatoes were bought from Xiaodian district, Taiyuan city in Shanxi Province. Pathogens including *B. cinerea*, *A. tenuissima*, and *F. moniliforme* were isolated from diseased tomatoes, identified by Henle–Koch law and molecular methods, and preserved in the laboratory.

Experimental methods

Isolation and selection of antagonistic bacteria from the surface of tomatoes

Tomatoes were kept moist at room temperature for 20 days. According to the method of Janisiewicz and Roitman,[15] five randomly selected tomatoes were placed into a 1000-mL beaker and 0.2 M phosphate buffer was added. It was ensured that the surface of the buffer was higher than the tomatoes. Washing solution was discarded after being shaken at 100 rpm for 10 min, and later phosphate buffer was added followed by ultrasonic cleaning twice for 30 s. The solution obtained after washing was diluted to 10-, 100-, and 1000-fold, and 100 μL from each solution was smeared on potato dextrose agar (PDA) culture medium and cultured at 26 °C for 2–3 days; all single colonies were cultured on PDA plates using streaking inoculation method.

Single colonies on PDA culture medium were picked for shaking culture at 200 rpm and 28 °C for 24 h, and made into $1 \times l0^{10}$ cfu/mL suspension; the surfaces of cherry tomatoes were sterilized for 2 min and washed clean using tap water, and then air dried under sterile conditions. A wound (3 mm × 3 mm) was made on the surface of a tomato using a sterilized inoculation needle, 50-μL antagonistic bacterial suspension was inoculated into the wound 2 h later, and then 15 μL 1×10^6 spore/mL pathogenic spore suspension was inoculated. Sterile water was used as a control and tomatoes were air dried, placed in plastic boxes, and kept moist for culture at 26 °C. Rate of incidence was measured after 6 days (tomato had disease when obvious bacterial growth and decay were observed), and the measurements were repeated thrice for every 30 tomatoes.

Identification of antagonistic bacteria

DNA extraction

Sodium dodecyl sulfate-proteinase K lysis method was used for DNA extraction, cetyltrimethylammonium bromide was used for precipitation of cell fragment and polysaccharide, and isopropanol precipitation was used for extraction.

16S DNA gene amplification

Bacterial *16S rDNA* was amplified according to the method of Coenye et al.,[16] primers were as follows: 27 forward: 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 1541 reverse: 5′-AAG GAG GTG ATC CAC CC-3′. Polymerase chain reaction (PCR) amplification condition was as follows: pre-denaturation was performed at 94 °C for 5 min; denaturation at 94 °C for 40 s; annealing at 55 °C for 50 s; extension at 72 °C for 1 min 15 s; the total number of cycles was 35; termination was done at 72 °C for 10 min. PCR: 0.8 μL Taq (5 U/μL); 10 μL 10× PCR buffer (Mg^{2+} Plus); 8 μL deoxynucleotide triphosphate (dNTP) mixture (2.5 mM each); 2.5 ng template DNA; 2 μL primer F1 (10 μmoL/L); 2 μL primer R1 (10 μmoL/L); ddH_2O was supplemented up to 100 μL. DNA fragments were recovered and purified, and sequence measurement was performed by Shanghai Invitrogen Biotechnology Co. Ltd. in Beijing.

ropB gene amplification

ropB gene was amplified according to the method of Brady et al.,[17] forward primer of CM7-f: 5′-AACCAgTTCCgCgTTggCCTg-3′ and reverse primer of CM7-r: 5′-CCTgAACAACACgCTCggA-3′. PCR amplification reaction condition: pre-denaturation was performed at 95 °C for 5 min; denaturation at 95 °C for 35 s; annealing at 55 °C for 1 min 15 s; extension at 72 °C for 1 min 15 s; the total number of cycles was 30; termination was done at 72 °C for 7 min. *ropB* gene sequence reaction volume was 100 μL: 0.8 μL Taq (5 U/μL); 10 μL 10× PCR buffer (Mg^{2+} Plus); 8 μL dNTP mixture (2.5 mM each); 2.5 ng DNA template; 2 μL F1 primer (10 μmol/L); 2 μL R1 primer (10 μmol/L); ddH_2O was supplemented up to 100 μL. DNA fragments were recovered and purified, and then sequence measurement was performed by Shanghai Invitrogen Biotechnology Co. Ltd.

Homology analysis and construction of dendrogram

After sequencing DNA genes, homological BLAST was compared in the National Center for Biotechnology Information database. MEGA4.0 neighbor-joining method was used for the construction of dendrogram, and 1000 times of similarity were calculated. Dendrogram node showed that the value of bootstrap was >50%.[18]

Analysis of physiological and biochemical parameters

Physiological and biochemical measurements were performed for two bacterial strains according to the 9th edition of *Bergey's Manual of Determinative Bacteriology*.[19]

Biocontrol effect of antagonistic bacteria Enterobacter cowanii B-6-1

Antibacterial activity of different concentrations of antagonistic bacterial solution

After *E. cowanii* was purified using bacteria-plate method, a single colony was inoculated into 100 mL lysogeny broth (LB) culture medium for shaking culture at 200 rpm and 30 °C for 24 h; concentration was measured using dilution plate method and then 1×10^5, 1×10^6, 1×10^7, 1×10^8, and 1×10^9 cfu/mL bacterial suspensions were prepared using sterile water. PDA culture medium was added and solidified on a culture plate of diameter within 9 cm, and 30 μL of different concentrations of antagonistic bacterial suspension was added separately and evenly smeared on the plate. After air drying, a diameter of 5 mm pathogenic fungi was inoculated at the center of the plate, and the antagonistic bacteria were replaced with sterile water as a control. Cultured at a constant temperature of 28 °C, the diameter of fungal colony was measured after 5–7 days. Inhibition rate was calculated and every treatment was repeated thrice, and experiments were also repeated thrice. Inhibition rate (%) = (dC − dT)/(dC − 5) × 100, where dC is the diameter (mm) of fungal colony in the control and dT is the diameter (mm) of fungal colony with different treatments.

Confrontation culture of E. cowanii B-6-1 strain and pathogenic fungi after tomato harvest

After antagonistic bacterium B-6-1 strain was inoculated in a straight line at a distance of 2.25 cm from the center on a diameter of 9 cm PDA plate using an inoculating loop according to the method of Walker et al.,[20] fungal pathogens such as *F. verticillioides*, *A. tenuissima*, and *B. cinerea* were inoculated at the center of the plate at a diameter of 5 mm, respectively, and cultured in the dark at 26 °C for 5–7 days. Measurement was performed until one side of pathogens grew toward the edge of the culture plate. Inhibitory effect of antagonistic bacterium on fungal pathogens was calculated. Experiments were repeated thrice and three repeats were performed each time. Inhibition rate (%) = (diameter of colony far from antagonistic bacterium − diameter of colony close to antagonistic bacterium)/diameter of colony far from antagonistic bacterium × 100.

Biocontrol effect of B-6-1 strain on B. cinerea

Healthy tomatoes were picked and their surfaces were sterilized using 2% NaClO. A wound (3 mm × 3 mm) was made at the equatorial site, with two wounds per fruit. Antagonistic bacterium *E. cowanii* B-6-1 strain was inoculated into the LB culture medium for shaking culture at 200 rpm and 30 °C for 24 h, and different treatment solutions were prepared as follows: A: zymotic fluid of 1×10^7 cfu/mL; B: zymotic fluid of 1×10^8 cfu/mL; C: zymotic fluid of 1×10^9 cfu/mL; D: supernatant collected after filtering bacterium using 0.22-μm filter membrane; E: bacterial suspension of 1×10^7 cfu/mL after centrifuging the zymotic fluid and washing twice with sterile water; F: culture medium of 1×10^7 cfu/mL sterilized at 121 °C for 20 min; Control: sterile water. A volume of 40 μL of

Table 1 – Incidence rate and the diameter of lesion after inoculation of the wounds of tomatoes with antagonistic bacteria and yeast against *B. cinerea*.

Strains	Incidence rate (%)	Diameter of lesions (mm)
CK	100.00 ± 0.00a	4.27 ± 0.15a
B-1	0.00 ± 0.00f	0.00 ± 0.00f
B-6-1	0.00 ± 0.00f	0.00 ± 0.00f
B-12	4.67 ± 1.76f	0.90 ± 0.20ef
B-13	41.33 ± 7.08cd	2.50 ± 0.29 cd
B-15	71.04 ± 4.33b	3.43 ± 0.23abc
B-20	28.67 ± 3.47de	1.78 ± 0.12de
Y-7	75.34 ± 2.91b	3.67 ± 0.24ab
Y-10	63.50 ± 2.88bc	2.89 ± 0.43bc
Y-18	11.98 ± 3.61ef	0.93 ± 0.18ef
Y-19	48.57 ± 3.17cd	2.87 ± 0.07bc
Y-12	0.00 ± 0.00f	0.00 ± 0.00f

SD = standard deviation.
Data in the table are expressed as mean ± SD. Different lowercase letters in the same row indicate significant difference at $P < 0.05$ level by Duncan's new multiple range test ($P < 0.05$).

the above solution was inoculated into the wound of tomatoes, air dried, and 2 h later 15 μL 1×10^5 spore/mL of *B. cinerea* spore suspension was inoculated, and stored moist at 26 °C for 6 days. The incidence of diseases was recorded and calculated, which was repeated thrice for every 20 tomatoes. Grading criteria for fruit diseases[21] were as follows: Grade 0, no disease; Grade 1, lesion area accounts for <5% of fruit area; Grade 3, lesion area accounts for 6–10% of fruit area; Grade 5, lesion area accounts for 11–25% of fruit area; Grade 7, lesion area accounts for 26–50% of fruit area; Grade 9, lesion area accounts for >50% of fruit area. Disease index = $\sum$(the number of rotten fruits at all levels × the representative value of the grade)/(the total number of fruits × the highest representative value) × 100; control efficiency = (control disease index-treatment of disease index)/control disease index × 100.

Statistical analysis

Statistical analysis of experimental data was analyzed using SAS9.0 software. Analysis of variance was used for Duncan multiple variance analysis. A value of $P < 0.05$ was considered significant.

Results

Selection of antagonistic bacteria

After comparison and identification of the tomato surface, the rate of incidence of lesion in the control group was 100%. Compared with the control, 11 strains (6 bacterial and 5 yeast) isolated had significant antagonistic effect ($P < 0.05$; Table 1). Three strains had the best antagonistic effect and they were B-1, B-6-1, and Y-12. After treatment with B-6-1 strain, the incidence rate was 0% and can completely inhibit *B. cinerea* (Fig. 1).

Identification of antagonistic bacterium

Colonial morphology

Morphological features of B-6-1 strain are as follows: The body of B-6-1 strain was short, rod-shaped, with a size of 0.6–1.0 μm × 1.2–3.0 μm. B-6-1 strain on the culture plate appeared round with irregular edges, light yellowish, and semi-transparent, with a bulged surface and was smooth and appeared semi-liquid.

Physiological and biochemical features

B-6-1 strain was negative for Gram staining with anaerobic growth and it cannot hydrolyze starch but can hydrolyze nitrite reductase. Methyl red reaction was negative, contact enzyme growth test was positive, and oxidase reaction was negative. Results are shown in Table 2.

Homology analysis and dendrogram construction of 16S rDNA in B-6-1 strain

The whole length of *16S rDNA* amplified using 27F and 1541R primers was 1441 bp. Homology analysis was performed after sequence BLAST, and DNAMEN software was used for multiple sequence alignment. Homology of sequences of B-6-1 strain and type strain *E. cowanii*^{T} (AJ508303), *Salmonella subterranea* FRC1S, *Salmonella enterica* ATCC, *Salmonella bongori* Br, and *E. cloacae* LMG was 99.6%, 98.4%, 98.1%, 97.9%, and 97.6%, respectively.

Bacillus licheniformis was used as an outgroup. MEGA4.0 software was used to construct B-6-1 and related *16S rDNA* dendrogram by neighbor-joining method (Fig. 2). B-6-1 and *E. cowanii* (registration number AJ508303) clustered at the same branch, and clustering bootstrap support rate was 97%; it was initially considered that this antagonistic strain was *Enterobacter*.

Homology analysis and dendrogram construction of ropB of B-6-1 strain

To accurately identify B-6-1 strain, *ropB* gene was further verified. The length of the fragment sequence of CM7 primer amplification was 752 bp. Homology analysis was performed after sequence BLAST, and DNAMEN software was used for multiple sequence alignment of *ropB* gene. Homology with *E. cowanii* was 98.9%, followed by homology with *E. cloaca* (CP002886) and *E. nimipressuralis* at 92.3%; homology with *E. cancerogenus* (AJ56694) was 92.2%, homology with *E. asburiae* (AJ543727) was 91.9%, homology with *E. nimipressuralis* (AJ566948) was 91.6%, and homology with *Escherichia coli* (JN707682) was the lowest at 91.4%.

Dendrogram of B-6-1 strain and *ropB* gene was constructed using neighbor-joining method (Fig. 3). B-6-1 strain and type strain CIP 107300 (registry number AJ566944) of *E. cowanii* clustered at the same branch, and clustering bootstrap support rate was 100%. It has been reported that homology of *16S rDNA* sequence of bacterial colonies reached up to >97%, and hence it can be considered as isogeny.[22] Therefore, combined with bacterial morphology, physiological and biochemical features, *16S rDNA* and *ropB* gene sequence analysis, B-6-1 strain can be identified as *E. cowanii*.

Fig. 1 – Inhibitory effect of antagonistic bacteria B-6-1 strain on *B. cinerea*.

Table 2 – Physiological and biochemical characteristics of B-6-1.

Characteristics	Results	Characteristics	Results
Catalase	+	Oxidase	–
Methyl red reaction	–	Nitrate reduction	+
β-Galactosidase	+	Arginine double hydrolysis	–
Lysine decarboxylase	–	Ornithine decarboxylase	–
Citric acid use	+	H_2S production	–
Urease	–	Tryptophan deaminase	+
Indole production	–	VP reaction	+
Gelatinase	–	Inositol	+
Melezitose	–	Glycerin	+
Mannitol	+	Raffinose	+
Erythritol	–	Sorbitol	+
D-Arabinose	–	Starch	–
α-Methyl-D-mannose glycosides	–	Glycogen	–
α-Methyl-D-glucoside	–	Xylitol	–
N-Acetyl-glucosamine	+	Gentiobiosyl	+
Amygdalin	+	D-Turanose	–
Arbutin	+	D-Lyxose	+
Aesculin	+	D-Tagatose	–
Salicin	+	D-Fucose	+
Cellobiose	+	L-Fucose	–
Maltose	+	D-Arabitol	–
Lactose	–	L-Arabinitol	–
Melibiose	+	Gluconate	+
Sucrose	+	2-Keto-gluconate	w
Trehalose	+	5-Keto-gluconate	–
Inulin	–	β-Methyl-D-xyloside	–
L-Arabinose	+	D-Galactose	+
D-Ribose	+	D-Glucose	+
D-Xylose	+	D-Fructose	+
L-Xylose	–	D-Mannose	+
Adon alcohol	–	L-Sorbose	+
L-Rhamnose	+	Dulcitol	+

Physiological and biochemical characteristics index selected according to the 9th edition of *Bergey's Manual of Determinative Bacteriology*.

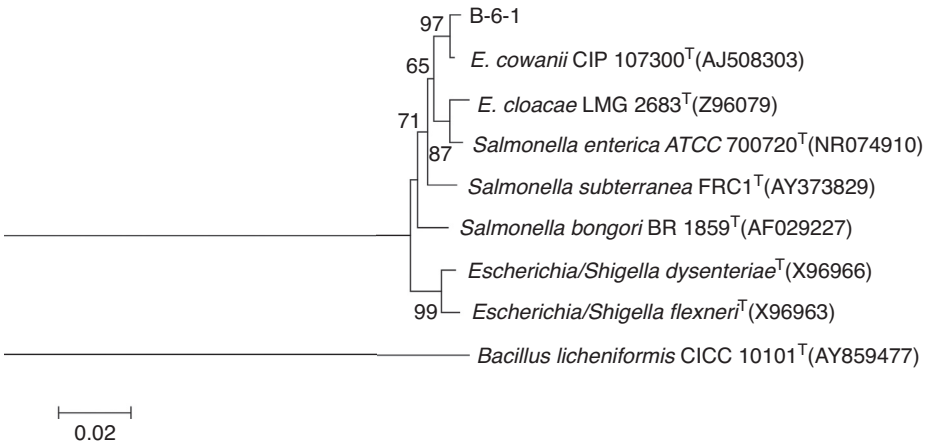

Fig. 2 – Dendrogram based on *16S rDNA* sequences of strain B-6-1 constructed using neighbor-joining method. Note: The number at the node means the percentage of occurrence in 1000 boot-strapped trees; the scale bar means 0.5% sequence difference; "T" in the upper right-hand corner indicates the type of strain.

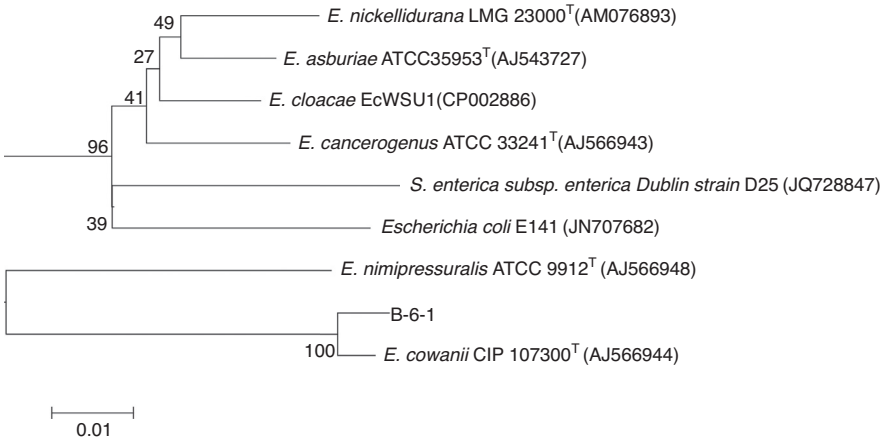

Fig. 3 – Dendrogram based on *ropB* gene sequences of strain B-6-1 constructed by neighbor-joining method. Note: The number at the node means the percentage of occurrence in 1000 boot-strapped trees; the scale bar means 0.5% sequence difference; "T" in the upper right-hand corner indicates the type strain.

Inhibitory activity of pathogens after tomato harvest by E. cowanii B-6-1

Effect of E. cowanii B-6-1 on the growth of three fungal pathogens after tomato harvest

All of different concentrations of B-6-1 bacterial suspension inhibited the growth of *F. verticillioides*, *A. tenuissima*, and *B. cinerea*. Inhibitory effects among different pathogens were different: inhibitory effect on *B. cinerea* was the strongest, followed by a stronger effect on *A. tenuissima*, and the weakest effect on *F. verticillioides*. At a concentration of 1×10^5 cfu/mL, the inhibition rate of *E. cowanii* against the mycelium of *B. cinerea* was 67.48%. The antagonistic bacterium at 1×10^9 cfu/mL could completely inhibit its growth (Table 3).

Table 3 – Inhibitory effect of different concentrations of antagonistic bacteria on three fungal pathogens ($P < 0.05$).

Concentration (cfu/mL)	Inhibition rate (%)		
	F. verticillioides	*A. tenuissima*	*B. cinerea*
1×10^5	46.31 ± 1.14b	67.48 ± 1.39b	75.67 ± 1.49b
1×10^6	48.12 ± 2.13b	68.29 ± 1.24b	76.53 ± 1.63b
1×10^7	48.72 ± 3.03b	69.38 ± 1.99b	78.00 ± 0.39b
1×10^8	53.33 ± 1.03b	74.53 ± 2.25ab	80.12 ± 1.90b
1×10^9	69.75 ± 2.08a	78.32 ± 1.91a	100.00 ± 0.00a

SD = standard deviation.
Data in the table are expressed as mean ± SD. Different lowercase letters in the same row indicate significant difference at $P < 0.05$ level by Duncan's new multiple range test ($P < 0.05$).

Confrontation culture of B-6-1 with fungal pathogens after tomato harvest

Results of confrontation culture of B-6-1 and fungal pathogens *F. verticillioides*, *A. tenuissima*, and *B. cinerea* are shown in Table 4. Growth of pathogens was significantly inhibited when three

Table 4 – Results of confrontation culture of antagonistic bacteria *E. cowanii* B-6-1 with fungal pathogens.

Types of pathogens	Inhibition rate (%)
A. tenuissima	34.59 ± 0.52
B. cinerea	35.79 ± 1.13
F. verticillioides	26.57 ± 0.95

SD = standard deviation.
Data in the table are expressed as mean ± SD. Different lowercase letters in the same row indicate significant difference at $P < 0.05$ level by Duncan's new multiple range test ($P < 0.05$).

types of fungal pathogens were close to the side of antagonistic bacterium, and obvious inhibition zone was observed at the junction between antagonistic bacterial and fungal pathogens. Inhibitory effects of B-6-1 on *B. cinerea* and *A. tenuissima* were stronger but the effect on *F. verticillioides* was weaker.

Biocontrol effects of different concentrations of B-6-1 antagonistic bacterial liquids on B. cinerae

After inoculation of treatment solution of different concentrations of *E. cowanii*, incidence of disease was calculated. With increasing concentrations of culture medium, the disease index of tomato botrytis disease was reduced. Disease index of tomato botrytis disease in the control was 93.38%, while that of 1×10^9 cfu/mL zymotic fluid was only 4.44%; biocontrol rate reached up to 95.24% and the incidence rate significantly decreased. On the other hand, biocontrol effects of bacterial suspension and zymotic fluid were similar; no significance was observed between the two treatments. Filtrate control effect reached 77.40%, and heat kill fluid on treatment had no obvious effect on tomato gray mold (Table 5).

Discussion

Currently, many antagonistic bacterial strains for inhibiting botrytis disease in post-harvest fruits have been used.[23–27] *Acremonium breve* has inhibitory effect on apple and grape fruit; *Bacillus subtilis* has inhibitory effect on botrytis disease of strawberry and cherry; *Candida guilliermondii*, *C. oleophila*, and *C. sake* can inhibit *B. cinerea* of pear, nectarine, and tomato fruits; *Cryptococcus laurentii*, *C. flavus*, and *C. albidus* inhibit botrytis disease of post-harvest apple, strawberry, tomato, and pear fruits; *Metschnikowia fructicola* and *M. pulcherrima* inhibit botrytis disease of apple and grape fruits; *Pseudomonas cepacia*, *P. corrugate*, and *P. syringae* have inhibitory effects on pear fruits and apple fruits; *Rhodotorula glutinis* inhibits botrytis disease of strawberry, pear, and apple fruits; *Trichosporon pullulans* suppresses *B. cinerea* of cherry fruits. The number of antagonistic bacteria on post-harvest tomato was less than other types of fruits. In this study, the discovery of *E. cowanii*, which has not been reported earlier, provides more choice for microbiological control of pathogens in post-harvest tomato.

In the ecosystem of fruit diseases and microbiology, when fruit diseases are severe, pathogens occupy a favorable position, whereas antagonistic bacteria is at inhibitory status. Therefore, fruits did not develop disease or have mild disease when effective antagonistic bacteria control the development of fruit diseases, and selection of antagonistic bacteria from these fruits is easier to produce better inhibitory effect of antagonistic bacteria.[28] In this experiment, fruits used for isolation of antagonistic bacteria were randomly selected and kept moist at room temperature for a period of time. Fruits without diseases were selected for the isolation of antagonistic bacteria to make antagonistic bacteria better adapt to the ecological environment of pathogens. Many researchers have selected antagonistic bacteria by combining *in vitro* and *in vivo* methods. *In vitro* selection is more convenient and rapid for examining the inhibitory effect of strains. However, in strains selected for inhibitory effect, *in vitro* condition might not produce the same inhibitory effect as *in vivo* experimental condition.[29] In this study, *in vivo* selection method was directly used to obtain good bacterial strain to avoid inconsistency between *in vitro* and *in vivo* experiments.

Enterobacteria widely exist in nature, and it has been reported that some strains in *Enterobacter* have very good biocontrol effects. *E. cloacae* has very strong antagonistic effects on *Pythium myriotylum in vitro*.[30] Yang et al.,[31] isolated *E. cloacae* from rice seedling root, spraying its zymotic fluid on the leaves of rice at booting stage can significantly improve antagonistic effect on bacterial blight, and biocontrol effect reached up to 38%. In 1995, Hinton and Bacon[32] found that *E. cloacae* from corn not only effectively colonized corn seedling but also distributed on the middle column and the cortex cells of maize root. The bacterium have a markedly antagonistic action on *F. moniliforme* and several other types of corn fungal pathogens. Yang[33] found *E. cowanii* during the isolation of 19

Table 5 – Inhibitory effects of various treatment solution of antagonistic bacteria *E. cowanii* on *B. cinerea* of post-harvest tomatoes.

Treatment	Disease index	Biocontrol effect (%)
Fermentation broth (1×10^7 cfu/mL)	71.14 ± 3.15 b	23.82 ± 1.81 d
Fermentation broth (1×10^8 cfu/mL)	48.89 ± 1.76 c	47.64 ± 1.02 c
Fermentation broth (1×10^9 cfu/mL)	4.44 ± 0.68 e	95.24 ± 2.07 a
Bacterial suspension (1×10^7 cfu/mL)	49.42 ± 3.59 c	47.08 ± 0.40 d
Filtration fluid	21.11 ± 1.79 d	77.40 ± 1.03 b
Heat kill fluid	94.23 ± 2.86 a	–
Control	93.38 ± 1.93 a	–

SD = standard deviation.
Data in the table are expressed as mean ± SD. Different lowercase letters in the same row indicate significant difference at $P < 0.05$ level by Duncan's new multiple range test ($P < 0.05$).

endophytic bacterial strains from different tissues and organs of cole, and studies have shown that this strain has certain inhibitory effects on fungal pathogens of cole. Fu[34] isolated endophytic bacterial strain *E. cowanii* fm50 from *Miscanthus floridulus* and *Cymbopogon caesius*, which have higher biological activities of nitrogenase as an enzyme and peroxidase, and promotes growth of rice. Brady et al.,[35] isolated *E. cowanii* strain from eucalyptus with bacterial blight and bud blight, He inferred that *E. cowanii* might exist in eucalyptus as an endophytic bacteria and has antagonistic effect. On the other hand, it is reported that glycoprotein in *Enterobacter cloacae* can inhibit normal growth of pulmonary cells.[36]

In this study, filtration liquid of *E. cowanii* B-6-1 can inhibit the occurrence of *B. cinerae*, which is similar to the results of a previous study on *E. cloacae*. Mukhopadhyay et al.,[29] found that in NB or PDB culture medium, filtration liquid of *E. cloacae* has inhibitory effects on *Rhizoctonia solani*, *Pythium myriotyum*, *Gaeumannomyces graminis*, and *Heterobasidion annosum*, demonstrating that antifungal metabolites exist in the liquid. *E. cowanii* B-6-1 heat killing bacterial solution did not have inhibitory effects, demonstrating that antibacterial substances do not tolerate high temperature. The similarity of antibacterial substance *E. cowanii* B-6-1 and antibacterial substance produced by *E. cloacae* was that *E. cloacae* filtration liquid did not have antibacterial activity after heating for 10 min at 60 °C or boiling for 5 min. On the other hand, inhibitory effects of the antagonistic bacterium correlated with the concentration of culture medium: the higher the concentration, the better the inhibitory effect, which is consistent with the results of previous research.[7]

In summary, B-6-1 strain was isolated from the surface of tomato, and identified as *E. cowanii* according to bacterial morphology, culture features, physiological and biochemical characteristics, and *16S rDNA* and *ropB* gene sequence analysis. *E. cowanii* B-6-1 not only has strong *in vitro* inhibitory effect on *F. verticillioides*, *A tenuissima*, and *B. cinerea* after tomato harvest but also effectively inhibits the occurrence of botrytis. The viability at low temperature and biocontrol efficiency in combination with other preservation measures and biocontrol mechanisms of *E. cowanii* are directions for future research.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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