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Biological Control of Postharvest Diseases of Chestnut Fruit by Microbial Antagonists-Endophytic Bacteria CE3

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24 endophytic strains were isolated from healthy Luotian chestnut tissue in China. 22 strains among the above 24 ones were bacterium, other strains were fungi. CE2, CE3 and CE8 showed effective biocontrol activities through flat confrontation method. Further, among the 3 strains CE3 had the best antagonistic effect. The inhibition zone width was 10.74 mm, 10.14 mm, 11.28 mm to *Cryphonectria parasitica*, *Penicillium expansum* and *Fusarium solani*. Based on the morphological, physiological characteristics and 16S rDNA sequence analysis, the strain CE3 was identified as *Bacillus cereus*. It's obvious that CE3 has the best inhibitory effect before infection of pathogenic bacteria, the best concentration is 10^9 CFU ml^{-1}. In order to enhance the bacteriostatic effect of *B. cereus*, we made a test, which was the *B. cereus* combine with auxiliary factor. The results showed that, When $CaC1_2$ and $MgC1_2$ were used in conjunction with CE3, there was significantly better control effect on *P. expansum* than individual uses of two adjuvants. The effect of treatment with CE3 alone had no significant difference. *B. cereus* CE3 controlled the growth of pathogenic fungi was enhanced by the addition of $CaC1_2$, $MgC1_2$. In addition, a combined use of carbendazim that had been diluted for 200,000 times and CE3 enabled the incidence rate of *A. niger* to drop significantly, with better inhibitory effect than exclusive use of CE3 or low doses of carbendazim.

Key word: *Castanea mollissima* Blume; Endophyte bacteria; Biological control; Isolation and identification.

The Chinese chestnut, belongs to the Fagaceae family, is a traditional nut and one of the most popular food across the world. The chestnut has a growing history of over 2000 years in china with an important role in the economy. The chestnut fruit is considered a high nutritional value food and it also has a long history of using as a tonic in traditional Chinese medicine[1, 2]. Chestnuts have a short shelf life due to their high moisture and sugar content[3]. In China, one third of the annual chestnut harvest is exported to Japan (Vossen, 2000); the remainder being mainly roasted and consumed locally, during which a large proportion is damaged, causing a great economic loss. This is mainly due to the high metabolic activity of the nuts and the epicarp characteristics that are porous and not ligniûed, which will cause a serious infections of fungi and bacterium during storage[4]. Postharvest decays of fruits and vegetables account for significant levels of postharvest losses. It is estimated that about 20-25% of the harvested fruits and vegetables are decayed by pathogens

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during postharvest handling even in developed countries(5). However, due to the public concern about environmental contamination and human health risks, biological control using microbial antagonists has shown potential as an alternative measure to synthetic fungicides for disease control. Postharvest biological control employing antagonistic microorganism has emerged as a promising alternative to synthetic fungicides in recent years[6]. In order to substitute synthetic fungicides, more environmentally friendly and harmless compounds should be developed as alternative methods for postharvest diseases[7]. This paper deals with the use of microbial antagonists for controlling postharvest diseases of chestnut fruits. the purpose of this study is to explore the green preservation methods for Chinese chestnut.

MATERIALS AND METHODS

Preparation of spore suspension of the pathogen

a) LB broth, pH 7.0, actidione (100 µg mL^{-1}).

b) PDA broth, pH 7.0, chloramphenicol (100 µg mL^{-1}).

Gause's No. 1 broth was improved by adding K_2NO_3 1g, 0.5g of KH_2PO_4, 0.5g of $MgSO_4$, 0.01g of $FeSO_4$, 0.5 g of NaCl, 20 g of starch, 1 ml of 3% potassium dichromate solution into every 300 ml when the sterilised broth was cooled off. The culture all autoclave at 121 °C for 20 min.

Isolation of the endophytic bacteria from chestnut

Healthy chestnuts from chestnut parks in Luotian(east longitude 115°26', northern latitude 30°37') were picked at random. These samples were placed in a sterilisation bag to start treatment right away in the laboratory, or placed temporarily at 4 °C and treated within 7 days. First, the samples were washed under running tap water, surface-sterilised by 0.1% $KMnO_4$ solution for 2-3 minutes, and finally rinsed 2-3 times in sterilised distilled water. After surface disinfection, they were placed in a dry, well-ventilated room. One hole (5 mm diameter and 3 mm depth) was perforated by a perforating machine on the equator of each chestnut's surface.

Morphological, physiological and biochemical characteristics of endophytic bacteria CE3

After activation, CE3 was inoculated on LB broth and placed upside down at 37 °C for 1-2 d. Colony characteristics were observed over separated single colonies for analysis of physiological and biochemical characteristics and observation of a Gram stain, as well as preliminary classification by referring to the "Manual for common bacteria identification" and "Bergey's Manual of Determinative Bacteriology".

Identification of endophytic bacteria CE3 in biolog system

According to Biolog's operating manual, a Gram-positive, anaerobic, spore-forming CE3 was subject to conventional activation treatment and incubated for 24 h in the designated broth. After incubation, the bacterial colonies were picked with toothpicks and bacterial suspension of certain concentrations was made up in normal saline. A pipette was used to transfer 150 µL of bacterial suspension to a Gram-positive, anaerobic GENIII microplate by Biolog Inc. The plate was incubated in darkness at 33 °C for 16 h. The microplate was measured by using the spectrophotometer, reading the bacteria's response characteristics "Atlas". By comparing to the standard strains in the database, the generic names and specific names of the sampled strains were determined.

Identification of endophytic bacteria CE3 by 16S rDNA gene sequences

After conventional activation of endophytic bacteria CE3, a small amount of bacteria was picked and the27F: 5'-AGAGTTTGATC CTGGCTCAG-3'; 1541R: 5'-AAGGAGGTGAT CCACCC-3' primers were used for polymerase chain reaction (PCR) to derive the 16S rDNA gene. Amplification of PCR was carried out in a 50 µL reaction volume containing 18 µL sterile ddH_2O, 25 µL 2×*Taq*mix, 1.5 µL Primer1, 1.5 µL Primer2 and 4 µL DNA. PCR conditions were as follows: 4 min initial denaturation at 94 °C, 30 s denaturation at 94 °C, 40 s annealing at 58 °C, 2 min extension at 72 °C, followed by 35 cycles, with a final extension at 72 °C for 10 min. PCR products were purified and sequenced bi-directionally by Shanghai Biological Engineering Co., Ltd, and the phylogenetic tree was constructed using MEGA5.1 by Neighbour-Joining method.

Preparation of CE3 filtrate sample

For the pre-treated chestnuts, 20 µL of the pathogen spore suspension was transferred by a pipette and inoculated into the chestnut wound as required. 20 µL of endophytic bacteria CE3 suspension was added 0 h, 6 h, 24 h prior to

inoculation, and 6 h and 24 h hours after inoculation, with saline as a control group. After air-drying the liquid added, the chestnuts were placed in a sterilisation kraft paper sack and stored in an incubator at 28 °C and relative humidity of 95% for 5-6 d. After that, the incidence was calculated by cutting the chestnuts for evaluation of the inhibitory effect. Three repeated experiments were set, each containing 20 chestnuts.

Statistical analysis

The data were analyzed by the analysis of the variance(ANOVA) in SPSS. Mean were separated by Tukeys HSD at $P<0.05$.

RESULTS

Isolation and screening of the endophytic bacteria from chestnut

Through dilution plate method, a total of 24 endophytic bacteria were isolated from healthy chestnuts, of which 2 strains were fungi and 2 strains were bacteria. No actinomycetes were isolated (Table 1). In dural culture assay, CE2, CE3 and CE8 could inhibit major chestnut pathogens in varying degrees. Among them CE3 gave the strongest antagonistic effect on *Cryphonectria parasitica*, *Fusarium solani* and *Penicillium expansum*. Its inhibition zone's diameter was 10 mm or more (Fig. 1), 10.74 mm, 11.28 mm and 10.14 mm respectively. CE8 had the best inhibitory effect against chestnut *Aspergillus niger*, with an inhibition zone of 5.25 mm in diamater. CE3's inhibitory effect on *Aspergillus niger* ranked only second to CE8. Compared comprehensively, CE3 was selected for further screening for antagonistic bacteria, a major disease of chestnuts.

Identification of CE3

The single bacteria CE3 were rod-shaped with flat ends (Fig. 2), colony round, ragged edges, smooth surface, moist, slightly opaque white, a diameter of about 1-2 mm, Gram-positive, spore-forming (Fig. 3).

Table 1. Antagonistic selection of 24 strains bacterium against 4 pathogens

Strain number	*Cryphonectria parasitica*	*Penicillium expansum*	*Fusarium solani*	*Aspergillus niger*
CE1	-	-	-	-
CE2	++	+	++	+
CE3	+++	+++	+++	+
CE4	-	-	-	-
CE5	-	-	-	-
CE6	-	-	-	-
CE7	-	-	-	-
CE8	+	-	+	++
CE9	-	-	-	-
CE10	-	-	-	-
CE11	-	-	-	-
CE12	-	-	-	-
CE13	-	-	-	-
CE14	-	-	-	-
CE15	-	-	-	-
CE16	-	-	-	-
CE17	-	-	-	-
CE18	-	-	-	-
CE19	-	-	-	-
CE20	-	-	-	-
CE21	-	-	-	-
CE22	-	-	-	-
CE23	-	-	-	-
CE24	-	-	-	-

Note: The control efficacy indicated with four grades respectively, +++ showed the inhibition zone diameters larger than 10 mm?++ is about 5-10 mm; + smaller than 5 mm; - showed no any bacteriostasis

Table 2. The identification results of endophytic bacteria CE3

Test item	Results16h
Control	-
dextrin	+
D-maltose	+
D-trehalose	+
D-cellobiose	-
Gentiobiose	-
Sucrose	+
D-(+)-Turanose	-
Stachyose	-
Negative control	+
pH 6.0	+
pH 5.0	+
D-raffinose	-
α-D-lactose	-
D-melibiose	-
β-methyl-D-glucose	-
D-salicin	+
N-acetyl-D-glucosamine	+
N- acetyl -²-D-mannosamine	-
N-acetyl-D-galactosamine	-
N-acetylneuraminic acid	-
1% NaCl	+
4% NaCl	+
8% NaCl	+
α-D-glucose	+
D-mannose	-
D-fructose	+
D-galactose	-
3-methyl-D- glucose	-
D-fucose	-
L-fucose	-
L-rhamnose	-
inosine	+
1% sodium lactate	+
Fusidic acid	-
D-Serine	+
D-Sorbitol	-
D-Mannitol	-
D-Arabitol	-
Inositol	-
Glycerinum	+
D-Glucose-6-phosphate	+
D-Fructose 6-phosphte	+
D-aspartic acid	-
D-serine	-
Troleandomycin	-
Rifamycin SV	+
Minocycline	+
Gelatin	+
Glycine-L-Proline	-
L-Alanine	-
L-Arginine	-
L-Aspartic acid	+
L-Glutamic acid	w
L-Histidine	+
L-Pyroglutamic acid	-
L-Serine	+
Lincomycin	-
guanidine hydrochloride	+
Sodium Tetradecyl Sulfate	-
Pectin	+
D-Galacturonic acid	+
L-galactonolactone	+
D-Gluconic acid	+
D-Glucuronic acid	+
Glucuronamide	+
Galactaric acid	-
Quininic acid	-
Saccharic acid	-
Vancomycin	-
Tetrazolium violet	-
Blue tetrazolium	-
p- hydroxyphenylacetic acid	-
Methyl pyruvate	-
D-Methyl lactate	+
L-Lactic acid	+
Citric acid	+
α-Oxoglutarate	+
D-Malic acid	-
L-Malic acid	+
Bromosuccinic acid	w
Nalidixic acid	+
LiCl	+
Potassium tellurite	+
Tween-40	-
γ-Aminobutyric acid	-
α- hydroxybutyric acid	-
β-hydroxy-D,L-butyrate	+
α-Tetruronic acid	w
Acetoacetic acid	+
Propionic acid	+
Acetic acid	+
Formic acid	+
Aztreonam	+
Sodium butyrate	+
Sodium bromate	+

Note: "+" indicate the positive or availability, "-" indicated negative or unavailability, "w" showed the boundary value.

Catalase test, oxidase test, arginine double enzymolysis, VP and methyl red reaction were positive, but endophytic bacteria CE3 could use citric acid, hydrolysed gelatin and reduction of nitrate. ²-galactosidase, lysine decarboxylase, H_2S and starch hydrolysis tests were negative.

The Biolog microbial identification system indicates the utilisation degree of different carbon sources and sensitivity to different chemicals of microorganisms to be detected according to colour changes of tetrazoles oxidation and reducing substance in the microplate. Through the Biolog GENIII identification of endophytic bacteria CE3 (Table 2), based on the comparison with Biolog strains database, CE3 after 16h incubation was identified as *B. cereus* with the SIM and DIS values of 0.921 and 4.246, respectively. The CE3 strain was 100% identified as *B. cereus.*

The 27F and 1541R primers were used to derive the 16S rDNA gene from the isolated endophytic bacteria CE3 gene set, and the amplified fragment was 1000-2000 bp in length (Fig. 4). After sequencing, the amplified PCR products were 1427 bp in length. The 16S rDNA gene sequences of CE3 were identified using the BLAST program from the GenBank data library, and CE3 showed the highest homology (100%) with *Bacillus cereus*.

Compared CE3 sequence with strains sharing high homology in the data library, and ClustalX was used for multiple sequence alignment, and the phylogenetic tree was constructed using MEGA5.1 by neighbour-Joining method (Fig. 5). Based on the phylogenetic tree, the CE3 fell into the same group with Bacillus cereus ATCC 14579. The results supported the above preliminary identification results. According to the morphology, physiological and biochemical characteristics, 16S rDNA gene sequences phylogenetic tree analysis, the entophytic bacterium strain CE3 was identified and named as *Bacillus cereus*.

The biological control of different concentration of CE3 on postharvest diseases

As shown in Table 3, different concentrations of *B. cereus* CE3 significantly affect the incidence of chestnuts. The incidence of major diseases of chestnuts decreases with an increase in CE3's concentration. When CE3's concentration is 10^7 CFU mL^{-1}, the incidence of each bacterium is

Table 3. Influence on inhibiting activity of Chestnut pathogens with different concentration of *B.cereus*

Cell suspension of CE3(CFU/mL)	*C. parasitica* Infection rate	*P. expansum* Infection rate	*F. solani* Infection rate	*A. niger* Infection rate
CK	90.00±5.00a	85.00±5.00a	91.67±2.89a	98.33±2.87a
10^7	83.33±7.64ab	76.67±7.64ab	85.00±5.00ab	91.67±10.40ab
10^8	76.67±7.64b	68.33±2.89b	80.00±5.00b	88.33±5.77ab
10^9	51.67±7.64c	43.33±7.64c	56.67±5.77c	86.67±7.63ab
10^{10}	40.00±5.00c	41.67±2.89c	56.67±2.87c	81.67±7.98b

Note: "±"are mean values of triplicate tests SD, the different alphabet of small letter indicated the discrepancy on 0.05 levels notable

Table 4. Influence about inoculation time of *B.cereus* on inhibiting activity of *Chestnut pathogens*

Inoculation time	*C. parasitica* Infection rate	*P. expansum* Infection rate	*F. solani* Infection rate	*A. niger* Infection rate
24h before pathogen	31.67±2.89f	28.33±2.89f	33.33±2.89f	61.67±2.89e
6h before pathogen	38.33±2.88e	35.03±5.00e	40.00±5.00e	70.00±5.00d
0h before pathogen	48.33±2.89d	41.67±2.89d	48.33±2.89d	76.67±2.89c
6h after pathogen	58.33±5.77c	53.33±2.89c	55.00±5.00c	81.67±2.89bc
24h after pathogen	61.67±2.89b	60.00±5.00b	63.33±2.89b	86.67±2.89b
CK	100.00±0.00a	100.00±0.00a	100.00±0.00a	100.00±0.00a

Note: "±"are mean values of triplicate tests SD, the different alphabet of small letter indicated the discrepancy on 0.05 levels notable

not significantly different from the control group. When CE3's concentration is 10^8 CFU mL^{-1} or more, incidence rates of Phytophthora fungus, penicillium expansum and Fusarium solani in chestnuts are significantly lower compared to the control group. When CE3's concentration is 10^{10} CFU mL^{-1}, the incidence of *Aspergillus niger* reduces 17% compared with control. There are significant differences while the concentrations of the other three groups show no significant differences with the control group ($p > 0.05$). The larger CE3 concentration is, the better inhibitory effect of chestnut diseases is. It can be speculated that higher concentrations of CE3 lead to more

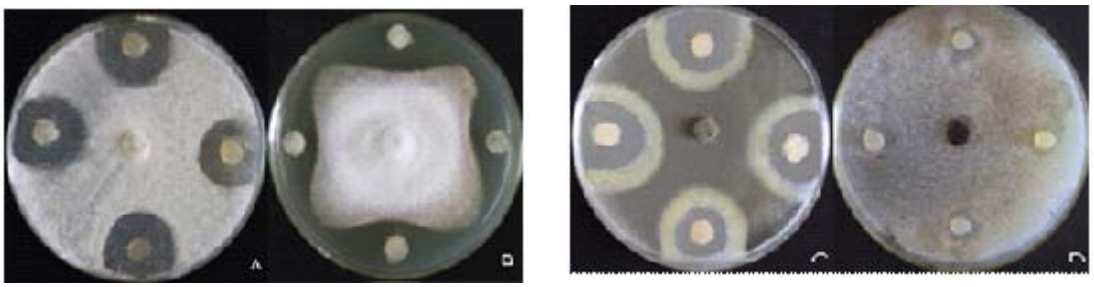


Fig. 1. A The bacteriostatic figure of CE3 against *C. parasitica*; B The bacteriostatic figure of CE3 against *F. solani*; C The bacteriostatic figure of CE3 against *P. expansum*; C The bacteriostatic figure of CE3 against *A. niger*

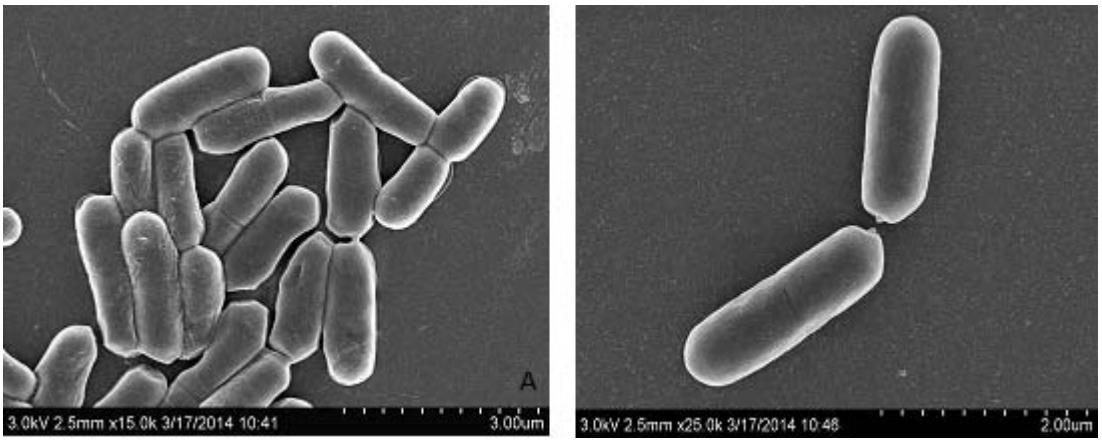


Fig. 2. The scanning electron microscopy (SEM) of CE3

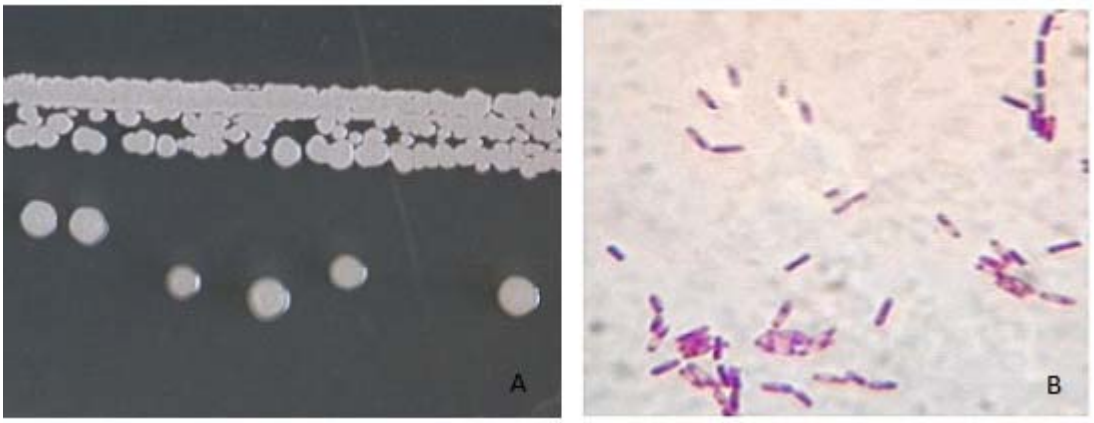


Fig. 3. The morphological characteristics of CE3

competitions with pathogens, which can occupy space and compete for nutrients on the wound, and therefore it has better prevention and treatment effect. However, when the concentrations of CE3 are 10^9 CFU mL^{-1} and 10^{10} CFU mL^{-1}, no significant differences could be observed in incidences of the four diseases. Taking into overall consideration, CE3 is best at the concentration of 10^9 CFU mL^{-1}.

The effect of different inoculation time of CE3 on postharvest diseases

As can be seen from Table 4, different inoculation time of *Bacillus cereus* CE3 have large

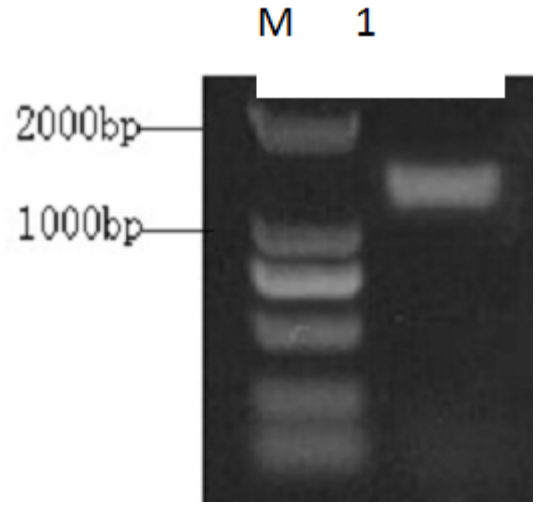


Fig. 4. The result of PCR to CE3 by current primer of 16S rDNA. lane M: DNA Marker(2000); lane 1: the amplification production of CE3

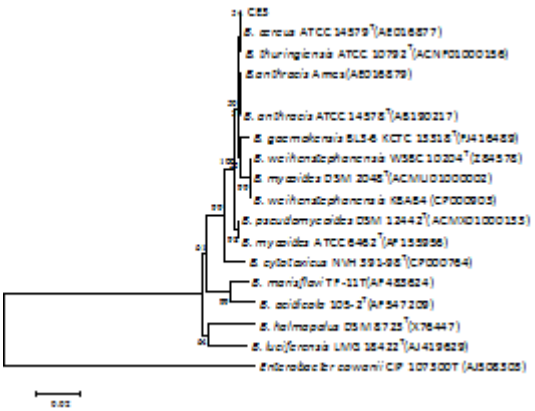
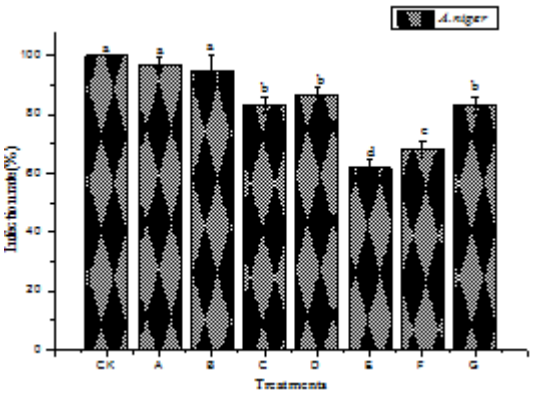


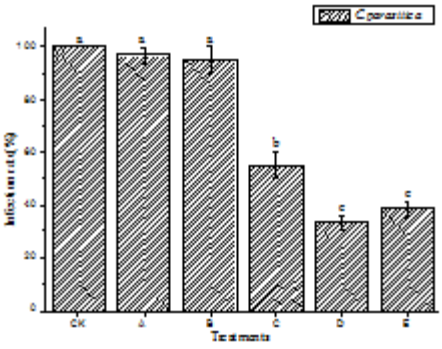
Fig. 5. Phylogenic tree of endophytic bacteria CE3 isolated from Chinese chestnut. Note: 0.02 means two changed per 100bases



CK: blank control using water; A: 0.2 M $CaCl_2$; B: 0.2 M $MgCl_2$; C: 0.2 M NaClO; D: 10^9 CFU mL^{-1} cell suspension of CE3; E: 0.2 M $CaCl_2$+10^9 CFU/mL CE3; F: 0.2 M $MgCl_2$+10^9 CFUmL^{-1} CE3; G: 0.2 M NaClO +10^9 CFUmL^{-1} cell suspension of CE3

The error bar are mean values of triplicate tests SD, the different alphabet of small letter indicated the discrepancy on 0.05 levels notable.

Fig. 6. The inhibition effect on *A.niger* when *Bacillus cereus* combined with chemical adjuvant.

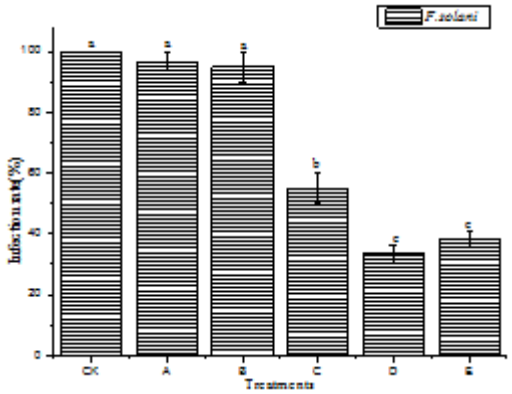


CK: blank control using water; A: 0.2 M $CaCl_2$; B: 0.2M $MgCl_2$; C: 10^9 CFU mL^{-1} cell suspension of CE3; D: 0.2 M $CaCl_2$+10^9 CFU mL^{-1} CE3; E: 0.2 M $MgCl_2$+10^9 CFU mL^{-1} CE3

The error bar are mean values of triplicate tests SD, the different alphabet of small letter indicated the discrepancy on 0.05 levels notable.

Fig. 7. The inhibition effect on *C.parasitica* when *Bacillus cereus* combined with chemical adjuvant

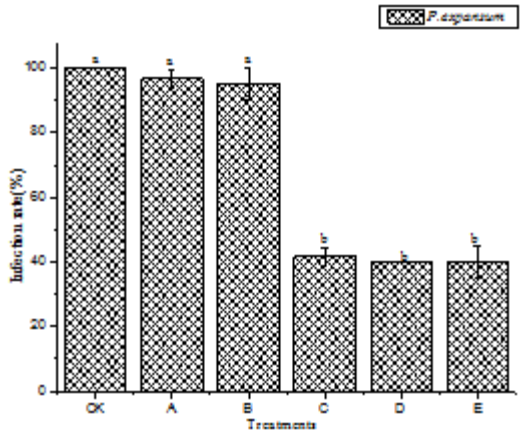
influences on control efficiency of major chestnut diseases. Inoculation of CE3 before pathogens has lower incidence than that after or simultaneous pathogens, and there are significant differences ($p<0.05$). Especially, inoculation of CE3 24 h before pathogens has the best activity against pathogens, and there are significant differences with control and other vaccination time ($p<0.05$), signifying that CE3 has the best inhibitory effect before infection of pathogenic bacteria.



CK: blank control using water; A: 0.2M $CaCl_2$; B: 0.2M $MgCl_2$; C:10^9CFU mL^{-1} cell suspension of CE3; D: 0.2 M $CaCl_2$+10^9 CFU mL^{-1} CE3 E: 0.2 M $CaCl_2$+10^9 CFU mL^{-1} CE3

The error bar are mean values of triplicate tests SD, the different alphabet of small letter indicated the discrepancy on 0.05 levels notable.

Fig. 8. The inhibition effect on *F.solani* when *Bacillus cereus* combined with chemical adjuvant.



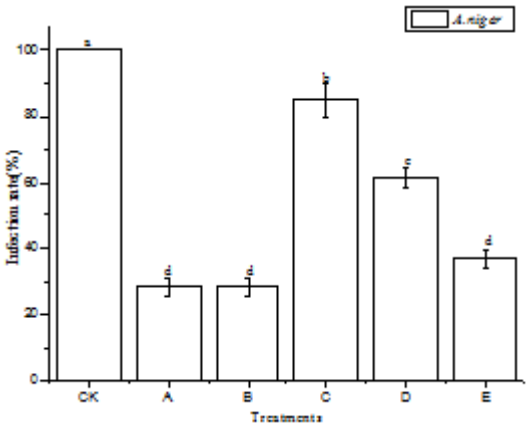
CK: blank control using water; A: 0.2 M $CaCl_2$; B: 0.2 M $MgCl_2$; C: 10^9 CFU mL^{-1} cell suspension of CE3; D: 0.2 mol/L $CaCl_2$+10^9 CFU mL^{-1} CE3 E: 0.2 M $MgCl_2$+10^9 CFU mL^{-1} CE3

The error bar are mean values of triplicate tests SD, the different alphabet of small letter indicated the discrepancy on 0.05 levels notable.

Fig. 9. The inhibition effect on *P.expansum* when *Bacillus cereus* combined with chemical adjuvant

The effect of biological control by CE3 binding several chemical adjuvant

Individual processing of three chemical adjuvants can reduce the incidence of chestnut *Aspergillus niger* (Fig. 6), in which treatment of 0.2 M $CaCl_2$ and $MgCl_2$ showed no significant differences from the control ($p> 0.05$), while 0.2 M sodium hypochlorite treatment was significantly different from the control ($p <0.05$). The combined treatment of CE3 and $CaCl_2$ and $MgCl_2$ in the chemical adjuvants had lower incidence than individual *B. cereus* treatment as well as individual treatment of two adjuvants, and there were significant differences ($p <0.05$). In other words, $CaCl_2$ and $MgCl_2$ could be used in conjunction with CE3, while CE3's combined treatment with sodium hypochlorite and sodium hypochlorite individual treatment had quite the same incidence rates, indicating that sodium hypochlorite had certain inhibiting effect on CE3 but could not enhance CE3's control efficacy, so sodium hypochlorite could not be used in conjunction with CE3.



CK: Blank control using water; A: Chlorothalonil diluted for 200,000 times B: Chlorothalonil that had been diluted for 200,000 times and the 10^9 CFU mL^{-1} cell suspension of CE3

C: 10^9 CFU mL^{-1} cell suspension of CE3 D: Carbendazim diluted for 200,000 times E: Carbendazim diluted for 200,000 times and 10^9 CFU mL^{-1} cell suspension of CE3

Fig. 10. The inhibition effect on *A.niger* when *Bacillus cereus* combined with low-dose chemical fungicides

Control efficacy experiment of *Cryphonectria parasitic* and *Fusarium solani* (Fig. 7 and 8) showed that individual treatment of 0.2 M $CaCl_2$ and 0.2 M $MgCl_2$ could inhibit chestnut *C. parasitic* and *F. solani*, but with unobvious effect. When these two adjuvants were used together with *B. cereus* CE3, there was better control efficacy against *C. parasitic* and *F. solani* and there were significant differences ($p < 0.05$).

From the experimental results (Fig. 9), as compared with the sterile water control, individual uses of 0.2 M $CaCl_2$ and $MgCl_2$ had no substantial inhibitory effect on *P. expansum*. When these two adjuvants were used in conjunction with CE3, there was significantly better control effect on *P. expansum* than individual uses of two adjuvants. The effect of treatment with CE3 alone had no significant difference ($p > 0.05$).

As can be seen from Fig. 10, while individually using chlorothalonil that had been diluted for 200,000 times, incidence of chestnut *Aspergillus niger* was 28.33%, a 71.67% decline compared with the incidence of the sterile water control, and there was significant difference. However, when combining the use of chlorothalonil and *B. cereus* CE3, the incidence of chestnut *A. niger* was quite equal to exclusive treatment of chlorothalonil, indicating that chlorothalonil could inhibit CE3, so the two could not be combined. Individually using carbendazim that had been diluted for 200,000 times and CE3 cell suspension in treatment of chestnuts had inhibitory effect on chestnut *A. niger*, but the incidence rates were 85.00% and 61.67%, respectively. After combination, both incidences dropped to 36.67%. Through Duncan's multiple analysis, there was a significant difference ($p < 0.05$), illustrating that CE3 could be combined with a low concentration of carbendazim.

DISCUSSION

Isolation and identification of endophytic bacteria from chestnut

Due to environmental influence and other factors, antagonistic microorganisms isolated from soil and other environments typically have dissatisfactory control efficacy of diseases. Endophytic bacteria refer to a class of microorganisms living in plant tissues or tissue gaps but causing no harm to plants or plant diseases. Endophytic bacteria are less influenced by the external environment, so they can grow effectively inside and on the surface of fruits and vegetables, and can better adapt to the fruit and vegetable storage environment than microorganisms in external environments. Moreover, the existence of microbial-mediated induced resistance has also been widely demonstrated for endophytic fungi and bacteria[8]. In recent years, a large number of endophytic bacteria were screened for disease control in fruits and vegetables. For example, Xie[9] isolated 96 strains of endophytic bacteria from 6 plants such as Codiaeum variegatum and Alpinia oxyphylla, among which the endophytic bacteria YXG2-3 presented the best inhibitory effect on *Fusarium oxysporum* in the dish antagonistic effect test, with antibacterial band of 20 mm and 63.8% control efficiency in pot experiments. Lai[10], isolated an endophyte from the root tissue of *Sophora tonkinensis*, was identified as *Paenibacillus polymyxa*, which was found to be highly effective in reducing postharvest green mold decay of citrus fruit caused by *Penicillium digitatum*. Wang[11], on the other hand, isolated 28 strains of endophytic bacteria from *Murraya paniculata*, in which HBS-1 strain had inhibition rates of 42.0% and 50.0% respectively on mango Anthracnose and *Diplodia natalensis*, and HBS-fermentation broth of 50-fold dilution had equal inhibition efficacy with sterilising agent TBZ of 800-fold dilution.

In this study, a total of 24 endophytic bacteria were isolated from healthy chestnuts, of which 2 strains were fungi and 2 strains were bacteria. In dural culture assay, CE2, CE3 and CE8 could inhibit major chestnut pathogens in varying degrees. Among them CE3 gave the strongest antagonistic effect on *C. parasitica*, *P. expansum* and *F. solani*. Its inhibition zone's diameter was 10 mm or more, 10.74 mm, 10.14 mm and 11.28 mm respectively. According to the morphology, physiological and biochemical characteristics, 16S rDNA gene sequences phylogenetic tree analysis, the entophytic bacterium strain CE3 was identified and named as *Bacillus cereus*. To our knowledge, this is the first report about antagonistic *B. cereus* CE3 as a promising biocontrol agent against postharvest fruit of chestnut.

The biological control and synergia of CE3 on postharvest diseases

The in vitro inhibition efficacy test on major chestnut diseases shows that within the range of 10^7-10^{10} $CFUmL^{-1}$, the greater *B. cereus* CE3 concentration is, the better inhibition of main chestnut diseases have, which may be because greater number of CE3 led to greater advantages in competition for nutrients and survival space. In addition, the experiment also verified that different inoculation time of CE3 had large influences on control efficiency of major chestnut diseases. Specially, inoculation of CE3 before pathogens has lower incidence than that after or simultaneous pathogens, and there are significant differences, signifying that CE3 had the best inhibitory effect before infection of pathogenic bacteria, and had unsatisfactory control efficacy on chestnuts that had been infected with pathogens.

In order to strengthen prevention and cure of chestnuts that had been infected with pathogens, other preventive measures were taken. Literature reports that a combination between metal salts like calcium and magnesium or low doses of chemical fungicides and antagonistic microorganisms can enhance the control effect of antagonistic microorganisms on postharvest diseases. For instance, Yang confirmed that treating nectarine with 0.2 M of $CaCl_2$ and bacteria suspension had better control efficacy against postharvest nectarine green mould than treating nectarine exclusively by $CaC1_2$ and bacillus subtilis. Besides, a combination of 4% Na_2CO_3 and *Bacillus subtilis* had better control efficacy than exclusive use of 4% Na_2CO_3(12). Our results show that treating chestnuts by combining CE3 with 0.2 M $CaC1_2$ and 0.2 M $MgC1_2$ had better control effects than exclusively by calcium chloride and magnesium chloride against four major postharvest diseases, and was also superior to exclusive treatment of CE3, and there was a significant difference. The combined treatment of sodium hypochlorite and CE3 had equal incidence with exclusive treatment of sodium hypochlorite, indicating that sodium hypochlorite had a certain inhibitory effect for CE3, but could not assist CE3 in prevention and treatment of postharvest disease. In the prevention test of Apple anthracnose, Li Miao and Wang Qian, *et al.* also found that apples inoculated with *Crytococcus laurentii* on the wound had lower incidence of anthracnose than the control group, while combining antagonistic yeast and calcium, Apple anthracnose could be prevented and cured better than exclusive use of antagonistic microorganisms and calcium. Our findings were consistent with the research by Miao Li *et al.*,[13]. At present, there are still controversies about the prevention mechanisms of antagonistic microorganisms strengthened with metal salts like calcium salts on postharvest diseases. For example, Mclaughlin *et al.* reported that the control effect of yeast cells enhanced by calcium on postharvest Penicillium and gray mould lied in the interaction of calcium and yeast metabolites on fruit wound, which did not involve a direct effect of calcium salts on apple gray mould, Penicillium and apple tissues[14]. On the contrary, Martin indicated that calcium could not only inhibit spore germination and germ tube extension, but the calcium ions could be combined with pectin in cell walls of fruits and vegetables and enhance the integrity and structure resistance of the cell walls, which greatly weakened pathogens' abilities to soften cell walls and invade fruits and vegetables. Mechanisms concerning the control effect of antagonistic microorganisms by metal salts strengthened with calcium salts and so on remain to be further investigated[15].

Experimental results show that a combined use of carbendazim that had been diluted for 200,000 times and CE3 enabled the incidence rate of *A. niger* to drop significantly, with better inhibitory effect than exclusive use of CE3 or low doses of carbendazim. Zhang Hongyin also reported that when low doses of iprodione were used in combination with C. laurentii, it could completely prevent pome Penicillium from occurring, completely preventing the incidence of Penicillium, so its inhibitory effect was significantly higher than exclusive uses of iprodione and C. laurentii, and fairly equal to the effect of conventional doses of iprodione[16]. CE3's combination with low-dose carbendazim can improve CE3's control effect, indicating that CE3 was resistant to carbendazim, and both had biocompatibility and could be combined. However, after using a combination of chlorothalonil and CE3, the incidence of chestnut *A. nige*r was fairly equal to that of chlorothalonil alone, showing that CE3 had no resistance to chlorothalonil and these two could not be combined.

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