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Screening and Control Efficacy of Endophytic Bacteria CE3 from *Castanea mollissima* Blume

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***Castanea mollissima*, as one of the important economic tree, is planted widely in China. In this work, a type of *Bacillus cereus* CE3 was isolated from healthy Luotian chestnut seed. From the colonization experiment of CE3 in chestnut seed, the antagonistic effect test and study of storage period on the seed induced resistance. The results showed that CE3 has antibiosis and induced resistance to the prevention mechanism of pathogenic bacterium after picking chestnuts. It can also increase the POD and PPO dense enzyme activity and reduce the MDA content caused by chestnut postharvest pathogenic fungi. Besides, the colonization of dynamic test on the surface of chestnut showed that CE3 had strong affinity on the surface of Chestnut. In consequence, the mechanism for *B.cereus* preventing and controlling postharvest diseases of Chestnut included two sides: antibiotic effect and inducible resistance. By further speculating competing nutrition space might be also one of the mechanisms.**

Key words: Chestnut; Pathogenic fungi; Endophyte; *Bacillus cereus*; Biocontrol.

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 during postharvest handling even in developed countries[1]. In developing countries, postharvest losses are often more severe due to inadequate storage and transportation facilities[2]. 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 yeasts has emerged as a promising alternative to synthetic fungicides in recent years[3]. Nevertheless, application of antagonistic microorganisms alone does not provide commercially acceptable control of postharvest diseases[4]. In order to substitute synthetic fungicides, more environmentally friendly and harmless compounds should be developed as alternative methods for postharvest diseases[5].

The Chinese Chestnut(*Castanea mollissima* Blume) is popular in East and southeast Asia because of its sweet taste. However, browning and postharvest diseases is a serious technical problem in chestnut processing[6]. This problem cause enormous economic loss in China and Asia. Recently, synthetic fungicides are primarily used to control postharvest diseases of chestnut. However, the global trend appears to shifting towards reduced use of fungicides on produce and hence, there is a strong public desire to seek safer and eco-friendly alternatives for reducing the decay loss in the

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harvested commodities[7]. In view of this, 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 chestnut.

MATERIAL AND METHODS

Preparation of spore suspension of the pathogen

Endothia parasitica(Murr), *Fusarium solani*, *Penicillium expansum*, *Aspergillus niger*, which keep on the PDA(Potato dextrose agar) culture at 25 °C, were isolated from the natural infected chestnut fruit in Hubei Luotian. Spore suspensions were prepared by flooding 7-days PDA cultures with sterile distilled water. Spore concentrations were determined with a hemacytometer and adjusted to the required concentrations.

The EC3(A type of *Bacillus cereus* Frankland) was isolated from the inside of chestnut fruits using method of Hua and identified by morphological, physiological experiments and ITS analysis[8]. The CE3 was cultured in 250-ml Erlenmeyer flasks with 30 ml of PDA culture on a gyratory shaker at 200 r/min for 24 h at 37 °C, then the cells were harvested by centrifuging at 7000 ×g for 10 min and resuspended in sterile distilled water. The cell suspensions were adjusted to concentrations of 1×10^6 or 1×10^8 CFU ml^{-1}, respectively, with a hemocytometer.

Preparation of CE3 filtrate sample

An appropriate amount of *B. cereus* CE3 liquid was added to a 10 mL centrifuge tube, and the tube was centrifuged at 13000×g for 10 min at a low temperature. The supernatant was collected in the centrifuge tube, and centrifuged again under the same conditions. The secondary supernatant was filtered using a 0.2 ¼m pore filter membrane to collect the final *B. cereus* CE3 filtrate sample.

Preparation of three kinds of treatment reagent: *B. cereus* CE3 original liquid (10^9 CFU/mL), CE3 bacterial suspension (10^9 CFU/mL), *Bacillus cereus* CE3 filtrate. twenty ¼L of the above treatment fluids were inoculated to the pretreated chestnut wounds separately, with sterile water to make the blank control. After this the fluids were placed for 2 hours before addition of 20 ¼L of the pathogenic fungi suspension. The chestnut fluids were air-dried for 2 hours and stored in a sterilized kraft paper bag in an incubator at 28 °C and 95% relative humidity. After storing for 5-10 d, the chestnuts were cut to calculate the number of pathogenetic ones in order to evaluate the inhibitory effect. The experiments were totally repeated for 3 times with 20 chestnuts for each time.

Measurement of enzyme activeties

All enzyme extract procedures were conducted at 4 °C. 2-3 gram of flesh samples from five fruits with 0.3g polyvinyl poly-pyrrolidone(PVPP)(SANGON, China) were ground with 10 ml of 0.1 M sodium phosphate buffer(pH 6.4) for POD(Peroxidase) and PPO (Polyphenol oxidase), 10 ml of 10% trichloroacetic acid, TCA buffer for malondialdehyde measured. The supernatants were used as the crude enzyme source to assay enzyme activities and malondialdehyde contents.

POD activity was carried out as described by Wang, with some modiûcation[9]. The reaction mixture consisted 1 ml crude extract and 3 ml reaction solution (50 ml of 100 mM sodium phosphate (pH 6.4), 0.028 ml of guaiacol, and 0.019 ml of 3% H_2O_2). The activity was determined by measuring the increase in absorbance at 460 nm.

PPO activity was determined according to Del's method[10] by adding 0.1 ml of enzyme preparation to 5.0 ml of catechol substrate (0.1 M catechol substrate, in 100 mM sodium phosphate, pH 6.4) and the increase in absorbance at 398 nm was measured immediately. The enzyme activity of POD and PPO were expressed in units (U) per microgram protein, where 1 unit was deûned as DA of 0.01 per minute.

Determination of MDA(Malondialdehyde) content: MDA was determined by TBA reaction as described by Heath method[11]. The MDA content was calculated using extinction coefficient of 155 $mMcm^{-1}$.

Treatments of the fruit

The chestnuts collected in the experiment were called "Bayuehong" or "Wukeli". They were free of pests, diseases and wounds, as picked from Luotian(east longitude 115°262 , northern latitude 30°372) Hubei

province, China. After washing with water to remove the surface dust, the chestnuts were soaked for 5 min with 0.1% potassium permanganate and then rinsed with clean water and air-dried for standby application.

Statistical analysis

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

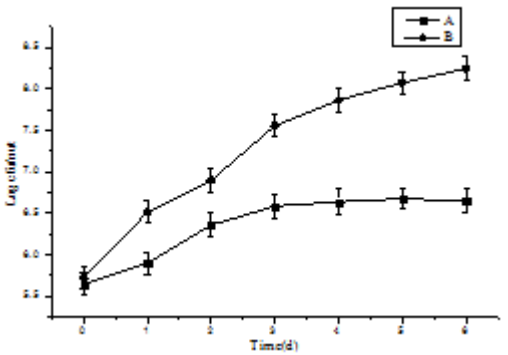
RESULTS

The colonization ability of CE3 on the chestnut

It can be seen from Fig. 1, the bacteria number in *B. cereus* CE3 on the chestnut surface first increased steadily and became constant afterwards, maintaining at the overall level of 10^7 CFU. CE3 can effectively colonize on the chestnuts, and its colonization ability on the chestnut surface can be enhanced significantly after adding chitosan membrane on the CE3 original liquid, presumably because chitosan forms a liquid membrane on the chestnut surface which wraps the bacteria and nutrients, thus enhancing the colonization ability on the chestnut surface.

The antibiosis of CE3 on pathogens on chestnut fruits

The treated *Bacillus cereus* CE3 was used to respectively perform the vivo bacteriostasis experiments on the four major postharvest diseases, as shown in Fig. 2. For chestnuts treated with CE3 original liquid, bacterial suspension, and filtrate, respectively, they had apparently lower incidence rates compared with the control sample. Among them, chestnuts treated with bacterial suspension had higher incidence of disease than the other two groups, and CE3 original liquid presented the best control efficiency of chestnut diseases. Although the control efficiency of CE3 filtrate was inferior to the CE3 original liquid, there were no significant differences of the incidence of four major diseases between these two kinds of treatments, indicating that CE3 can produce extracellular metabolites that inhibit the occurrence of diseases.



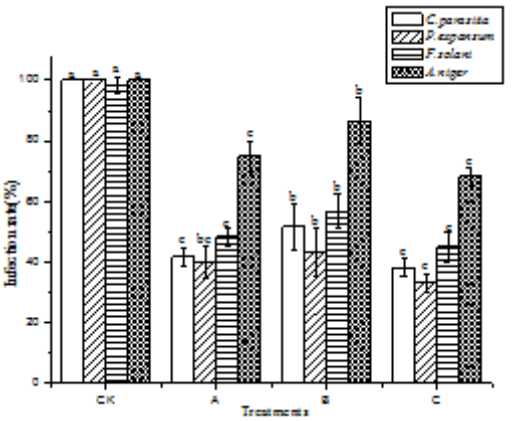
A: The culture medium of CE3 on 109 CFU/ml; B: The culture medium of CE3 on 109 CFU/ml plus 2% chitosan film.

Note: Values shown represent the mean reading from three treated sample and the error bars indicated the standard errors of the mean

Fig. 1 Population dynamics of B.cereus on the surface of Chestnut

The effect of CE3 on the activity of POD inside fruit

POD is one of the protective enzymes for plants to resist reactive oxygen damage. In addition, POD is also involved in the synthesis of lignin and promotes lignifications of the invaded tissues, thus forming a physical barrier to defense against the invading pathogen[12]. Fig. 3 showed that the content of POD in the control group was relatively stable. After treating chestnuts with pathogenic fungi and *B. cereus* CE3 alone, POD activity in chestnuts reached a peak level at 48 h,



CK: the control with water. A: Filtering medium on a concentration of 10^9CFU/mL CE3; B: The resuspension of CE3 on a concentration of 10^9 CFU/ml; C: The culture medium of CE3 on 10^9CFU/ml.

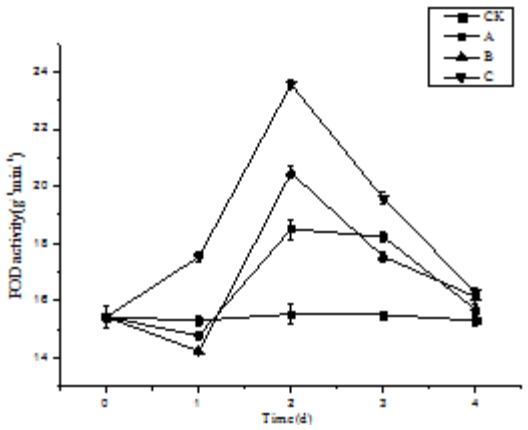
Note: Values shown represent the mean reading from three treated sample and the error bars indicated the standard errors of the mean; the different characters above the bar showed the test of Duncan's new multiple range on 0.05 level of Duncan.

Fig. 2 The inhibition effects on main chestnut pathogens with different disposed B.cereus

1.19 times and 1.32 times respectively of enzyme activity compared to the control group. Afterwards the enzyme activity continued to decline. After treating CE3 and pathogenic fungi for 48 h, the POD activity was higher than the control group and treatment of two bacteria alone. It was 1.54 times the enzyme activity of the control group. With the extension of the treatment time, the enzyme activity decreased and restored to its original level at 96 h, showing invisible differences with the control group. It is indicated that in the presence of pathogenic fungi, CE3 can effectively induce POD to strengthen activity, but efficacy duration is short.

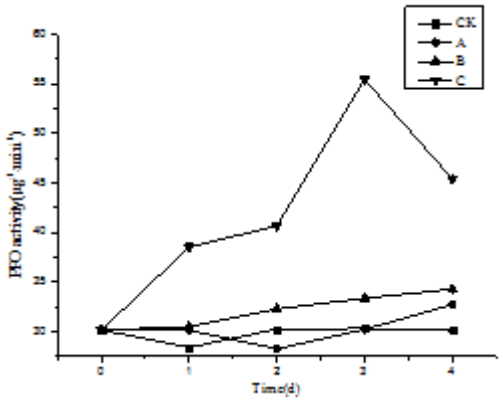
The effect of CE3 on the activity of PPO inside fruit

PPO (Polyphenol oxidase) forms quinines toxic to pathogens, thus directly playing an antiviral action. Further, it can also catalyze the formation of phenolic oxides and lignin, which indirectly inhibits pathogens' damage to plant cells. As shown in Fig. 4, PPO content in chestnut in the control group is relatively stable. In contrast, inoculation of pathogenic fungi solely and *B. cereus* CE3 can to a certain extent induce PPO activity in chestnuts to increase, but there is not much difference with the control group. After combining the treatment of CE3 and pathogenic fungi, PPO activity steadily and gradually increased in the first 48 h. After a sharp rise, it peaked at 72 h, which was 1.89 times the enzyme activity of the control group. 96 h after treatment, the enzyme activity remained 1.52 times compared with the control group, indicating that in the presence of pathogenic fungi, CE3 can effectively lead to an increase in PPO activity and have a certain period of preserving effect.



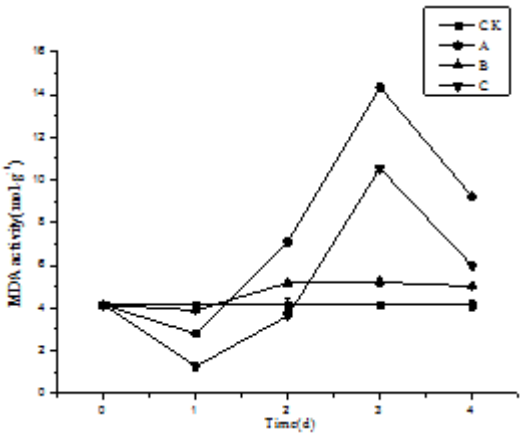
CK: The control treated with water; A: Pathogenic bacteria; B: Bacillus cereus CE3 C: B. cereus CE3 plus pathogenic bacteria. Note: Values shown represent the mean reading from three treated sample and the error bars indicated the standard errors of the mean.

Fig. 3 The influences on chestnut POD activity with different treated antagonistic microbes



CK: The control treated with water; A: Pathogenic bacteria; B: Bacillus cereus CE3 C: B. cereus CE3 plus pathogenic bacteria. Note: Values shown represent the mean reading from three treated sample and the error bars indicated the standard errors of the mean.

Fig. 4 The influences on chestnut PPO activity with different treated antagonistic microbes



CK: The control treated with water; A: Pathogenic bacteria; B: *Bacillus cereus* CE3; C: *B. cereus* CE3 plus pathogenic bacteria.
Note: Values shown represent the mean reading from three treated sample and the error bars indicated the standard errors of the mean.

Fig. 5 The influences on chestnut MDA coment with different treated antagonistic microbes

The effect of CE3 on the contents of MDA inside fruit

When plants are subjected to damage, accumulation of reactive oxygen species results in peroxidation in unsaturated fatty acids in the membrane, thus causing an increase in lipid peroxidation products MDA content. The membrane permeability increases, so MDA content can reflect the degree of plant damage[13]. As can be seen from the experimental results (Fig. 5), MDA content in the blank control group was relatively stable; at 48 h after inoculation of the pathogenic fungi, MDA concentration sharply rised, peaking at 72 h, which was 3.59 times the MDA content in the control group, signifying that chestnuts' damage extent deepened and membrane permeability increased; after chestnuts were subjected to *B. cereus* CE3 treatment, the MDA content slowly increased from 24 h, and has been maintained at a low level since 48 h, indicating that CE3 had almost no damage or no obvious damage to chestnuts during the measurement time. After combining the two bacteria, compared to treatment with the pathogen alone, MDA concentrations were lower at each time point, and after 96 h, MDA content showed little difference from the blank control, indicating that CE3 can effectively reduce increasing MDA content induced by pathogen, so that chestnuts could remain relatively stable physiological state

DISCUSSION

From the colonization experiment of *B. cereus* CE3 in chestnuts, the antibiotic effect tests and research of storage period on Chestnuts' induced resistance, we believe that CE3 has antibiosis and induced resistance to the prevention mechanism of disease after picking chestnuts. We speculate that there is also the possibility of competing for nutrients and space.

The colonization ability of endophytes is an important indicator of its biocontrol effect size[14]. It can be seen from the colonization dynamics of endophyte *B. cereus* CE3 on chestnut surfaces, CE3 has a strong affinity for chestnut surface. It can rapidly proliferate in a short time, and maintain the stability of population quantity, demonstrating that *B. cereus* CE3 can adapt to the complex environments of chestnut surface, which is closely related to its thermal tolerance, strong resistivity, fast growth and other characteristics. Precisely because of its strong resistivity, Bacillus has attracted wide attention from researchers home and abroad and has been successfully applied in agricultural production. For example, with a certain amount of water, the Bacillus subtilis strain QST713, which was developed by a U.S. company in 1995, was sprayed on the leaf surface. After large-scale demonstration and promotion, it has been proved to be able to effectively prevent blight disease and mycosis of some plants. Bacillus subtilis added with water aqua which was developed by Suke Agro-chemical of Jiangsu Province Co., Ltd. in our country has achieved over 80% of control efficiency to rice sheath blight disease and false smut[15]. The successful development of these antibiological inoculants favorably illustrates that such kind of antagonistic microbe has good potential for exploitation.

In antibiotic effect test of *B. cereus* CE3 to chestnut pathogens, CE3 original liquid, bacterial suspension and filtrate have varying degrees of control effects. Although CE3 filtrate has less ideal effect on preventing diseases than CE3 original liquid, there are no significant differences of incidence of the four major diseases under these two groups of treatment, signifying that CE3 can produce extracellular metabolites that inhibit the occurrence of diseases. CE3's production of extracellular metabolites is probably one of the mechanisms for controlling the chestnut postharvest diseases. The specific ingredients of such extracellular metabolites are yet to be studied.

B. cereus CE3's ability to induce host resistance has not been reported. The level of defense enzyme activity is an important indicator of disease resistance of plants. In the defense enzymes, POD is one of the protective enzymes for plants to resist reactive oxygen damage[16]. In addition, POD is also involved in the synthesis of lignin and promotes lignifications of the invaded tissues, thus forming a physical barrier to defense against the invading pathogen17. PPO forms quinines toxic to pathogens, thus directly playing an antiviral action[18]. Further, reactive oxygen metabolism loses balances when plants are subjected to damage, and MDA content can reflect the degree of plant damage[13]. The experimental

results show that stimulated by major postharvest pathogens, CE3 can significantly enhance the defense enzymes of POD, PPO in the chestnuts, enhance their resistance to pathogens, and can also effectively reduce the increasing MDA level caused by major pathogens after chestnuts are picked, so as to maintain relatively stable physiological state. Therefore, *B. cereus* CE3's inducing disease resistance of chestnuts is another mechanism for prevention of chestnut postharvest diseases.

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