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Biocontrol of Gray Mold Decay in Pear by *Bacillus amyloliquefaciens* Strain BA3 and its Effect on Postharvest Quality Parameters

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Abstract

The economic losses caused by postharvest fruits diseases have attracted global attention. Traditional chemical fungicide could not meet the need of humans. In recent years, microbial agent which has begun to take the place of chemical fungicide comes into people's vision. The aim of this paper was to investigate the potential of *Bacillus amyloliquefaciens* strain BA3 for its biocontrol capability on gray mold decay of pears and its effect on postharvest quality of pears. Compared with other treatments, the inhibition effect on gray mold of washed cell suspension of *B. amyloliquefaciens* was the best. Consequently it was utilized in subsequent experiments. Spore germination and germ tube length of *Botrytis cinerea* was 18.72% and 12.85 μm treated with BA3, while the control group was 62.88% and 30.44 μm. We confirmed that increase of the concentration of *B. amyloliquefaciens*, improved the efficacy of BA3 in controlling gray mold decay of pears. Colonization variation of BA3 in wounds of pears was recorded. To begin with, the populations of *B. amyloliquefaciens* increased rapidly and remained stable. On the fourth day, there was a declining trend , after that the population increased to 4×10^5 CFU/wound and remained stable. BA3 had no significant effect on mass loss, titratable acidity, firmness and total soluble solids of pears that were stored at 25°C for 7 days comparing with control group. However, the effect of *B. amyloliquefaciens* on ascorbic acid was significantly higher than that of the control group. Our study indicates that *B. amyloliquefaciens* has a potential as postharvest biocontrol agent on pears.

Key words: *Bacillus amyloliquefaciens*, *Botrytis cinerea*, biocontrol agents, gray mold, quality parameters

Introduction

Postharvest diseases are the primary damages of fruits during cultivation, transportation and storage. Postharvest decay of fruits has caused significant levels of economic losses worldwide (Sugar and Basile, 2011; Luo *et al*., 2015). It is reported that in developed countries 20–25% of harvested fruits decay because of postharvest diseases and 50% in developing countries (Sharma *et al*., 2009; Lutz *et al*., 2013).

The pear is one of the world's cultivated fruits, more than 70 countries and regions all over the world produce pears. In recent years, the cultivation area and the production of pears increased rapidly in China, which has been ranked first in the world (Yang *et al*., 2015).

Gray mold decay spreads widely and is caused by *Botrytis cinerea* which becomes one of the most important postharvest pear diseases (Lutz *et al*., 2013). Currently, postharvest diseases are controlled mainly by chemical means (Zhang *et al*., 2008a). However, chemical control is unfriendly to the environment and even leads to hazardous effects on humans and the environment (Solanki *et al*., 2013). Due to the increasing public concern about the potential detrimental effects of synthetic fungicides abuse, it is necessary to explore the best pollution-free means to control postharvest diseases (Sansone *et al*., 2005; Liu *et al*., 2010; Liu *et al*., 2013). Microbial biocontrol agents show great potential for controlling postharvest decay of fruits as an alternative to chemical control (Fan and Tian, 2000).

Bacillus species can be isolated from a variety of substances and it has been proved that they can produce inhibiting substances acting against a wide range of pathogens (Arguelles-Arias *et al*., 2009; Solanki *et al*., 2012; 2013). The objective of this study was focused on the *Bacillus amyloliquefaciens* strain BA3 isolated from "douchi", controlling postharvest decay of pears by *B. amyloliquefaciens* and exploring: (1) biocontrol activity of *B. amyloliquefaciens in vitro*, (2) efficacy of *B. amyloliquefaciens* on conidial germination of

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B. cinerea, (3) population studies of *B. amyloliquefaciens* in wounds, (4) efficacy of *B. amyloliquefaciens* on control of gray mold decay of pears, (5) efficacy of *B. amyloliquefaciens* on quality attributes of postharvest pears.

Experimental

Materials and Methods

Pathogen inoculum. *B. cinerea* Pers, purchased from Guangdong Microbiology Culture Center (GIMCC), was cultivated on synthetic potato medium (extract of boiled potatoes, 200 ml; dextrose, 20 g; KH_2PO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 1.5 g; vitamin B1, 8 mg; agar powder, 20 g and deionized water, 800 ml; pH, 6 at 25°C for 7 days. To prepare a spore suspension of *B. cinerea*, the plates were flushed with 10 ml sterile distilled water, and then the spore concentration was determined with a hemacytometer and adjusted to required concentration.

Antagonist. *B. amyloliquefaciens* strain BA3, was isolated from "douchi" (a kind of Chinese soy products) and identified by BioSune Company (Shanghai, China). GenBank number is KF192921. BA3 was incubated on NA (Nutrient Agar: peptone, 5 g; beef extract, 30 g; NaCl, 5 g; agar powder, 15 g; 1000 ml distilled water; pH, 7.0–7.2) at 28°C. *B. amyloliquefaciens* strain BA3 was cultivated in 250 ml Erlenmeyer flasks with 50 ml of NB (NA without agar powder) which had been inoculated with a loop of the culture on a rotary shaker at 200 × g for 24 h at 28°C. Then four treatments of liquid cultures were used: (A) liquid cultures (LC): mentioned above; (B) autoclaved cultures (AC): autoclaving the liquid cultures for 20 min at 121°C; (C) Culture filtrates (CL): filtering centrifuged liquid supernatant of the antagonist through a 0.2 μm polycarbonate membrane filter; (D) washed cell suspension (WCS): the cells were harvested by centrifugation at 8 000 × g for 10 min, washed twice and then re-suspended with distilled water. The concentration of cells in the suspension was counted with a hemacytometer and adjusted to 1×10^8 CFU/ml with sterile distilled water and (E) sterile distilled water acted as the control (CK).

Fruits. Pear fruits (*Pyrus pyrifolia*) cultivar "huangguan" were harvested at commercial maturity, selected on uniformity size without physical injuries or infections. Steep the fruit for 2 min in 0.1% sodium hypochlorite to sterilize and air dry before being wounded.

***In vitro* antagonism.** To estimate the interactions between the antagonist and the pathogen *in vitro*, we coated 200 μl spore suspension of *B. cinerea* on synthetic potato medium plates with a glass rod evenly. Then cut a 3-mm-diameter disk from the plates and added 100 μl A-E suspensions of treatments into each well. One hour later, the plates were incubated at 28°C (Liu *et al.*, 2010). The inhibition zone diameters were recorded after 7 days. Each test consisted of three replicate trials of 3 plates. The test was repeated three times.

Efficacy of BA3 on conidial germination of *B. cinerea*. The efficacy of BA3 on spore germination and germ tube elongation of *B. cinerea* was assessed in potato dextrose broth (PDB) (Feng *et al.*, 2011). One hundred μl spore suspension of *B. cinerea* (1×10^6 spores/ml) was added to glass tubes containing 4.8 ml of PDB. One hundred μl quantity of 1×10^8 CFU/ml of *B. amyloliquefaciens* suspension or sterile distilled water (as control) was added into each tube, respectively. After 12 hours' incubation at 28°C on a rotary shaker (200 × g), a total of 100 spores per replicate were observed microscopically with a light microscope and at least 5 microscope fields were observed. Conidia were considered germinated when the germ tube length was equal to or longer than the conidia length (Lutz *et al.*, 2013). All treatments consisted of three replicates, and experiments were repeated three times.

Population studies of *B. amyloliquefaciens* strain BA3 on fruit wounds. A uniform 5 mm deep and 3 mm wide wound was made at the equator of each fruit (put on its side) using the tip of a sterile inoculating needle. We injected 20 μl cell suspension of BA3 (1×10^8 CFU/ml) into the wound of each pear. Then the treated pears were cultivated at 25°C (90% relative humidity). Population of BA3 was recorded after being incubated for 0 (2 h after treatment), 1, 2, 3, 4, 5, 6 and 7 days, respectively. Wounded tissue was removed with an sterile 7 mm (internal diameter) cork borer and ground with an autoclaved mortar and pestle in 50 ml of sterile 0.85% sodium chloride solution. Serial tenfold dilution was made and 0.1 ml of each dilution was spread in NA. The plates were incubated at 28°C for 2 days and the colonies were counted. Population densities of *B. amyloliquefaciens* BA3 were expressed as $\log_{10}$ CFU per wound. There were three single fruit replicates per treatment, and the experiments were repeated three times (Yu *et al.*, 2012).

Efficacy of BA3 in controlling of gray mold decay of pears. A uniform 5 mm deep and 3 mm wide wound was made at the equator of each fruit (put on its side) using the tip of a sterile inoculating needle. The cell suspension of BA3 was adjusted to gradient concentration consisting of 1×10^6, 1×10^7, 1×10^8 CFU/ml with sterile distilled water by hemacytometer, respectively. We added thirty microliters washed cell suspension of 1×10^6, 1×10^7, 1×10^8 and 0 (as control) CFU/ml into each wound, respectively. After two hours, we injected fifteen microliters of spore suspension of *B. cinerea* (1×10^5 spores/ml) to each wound. Treated fruits were stored at 25°C for 7 days or 0°C for 30 days after air-drying. Infection rates and lesion diameters on fruits were measured. There were three replicate trials of

10 fruits per treatment with complete randomization in each test. The test was repeated twice.

Quality parameters of postharvest pears treated with BA3. To appraise the effect of *B. amyloliquefaciens* on quality attributes of postharvest pear, the pear fruits were soaked in washed cell suspensions (1×10^8 CFU/ml) or sterile distilled water as a control respectively for 30 second, and then air-dried. The treated fruits were stored in artificial climate chamber with polyethylene-lined plastic boxes to retain high relative humidity at 25°C for 7 days. After storage, quality attributes were measured. Quality attributes of postharvest treated pears were made on three replicates of five fruits each, the test was repeated twice.

Mass loss. The weight of pear was measured by a JA31002 balance (± 0.01 g) (Shanghai Jingping Balance Instruments, China) before treatment (A) and after storage (B), respectively, and the mass loss was calculated as (A–B)/A (Zhang *et al*., 2008b).

Ascorbic acid. The 2, 6-dichloroindophenol titrimetric method (AOAC) was used to determine the ascorbic acid content of pressed fruit juice. Results were expressed as milligrams of ascorbic acid/100 g sample (AOAC, 1995).

Titratable acidity. Titratable acidity was determined by the method described by Özden and Bayindirli (2002). Titratable acidity was calculated as the percent of malic acid.

Fruit firmness. Firmness values of each pear were measured at three points. Insert a fruit ripeness tester (Wagner Instruments) by 90° at the equator of each fruit after the removal of 1 mm thick slice of peel. The firmness of each pear was measured three times on different sides.

Total soluble solids (TSS). We measured total soluble solids (TSS) with a hand-held refractometer, and recorded the refractive index of the same juice. The results were expressed as percentages (g per 100 g fruit weight) (Luo *et al*., 2015).

Statistical analysis. Statistical analyses were performed with SPSS version 19.0. The data were analyzed by analysis of the variance (ANOVA). Statistical significance was assessed at P-value < 0.01 and Duncan's Multiple Range Test was used to separate means.

Results and Discussion

Microorganism is a kind of useful biocontrol agent that can inhibit several pathogens fungi which cause postharvest diseases of fruits (Chen *et al*., 2009; Li *et al*., 2011; Askarne *et al*., 2012; Solanki *et al*., 2012; Yu *et al*., 2012; Hu *et al*., 2015). But to our knowledge, there is little information concerning about the effect of *B. amyloliquefaciens* on controlling postharvest gray mold decay of pears and the influence of *B. amyloliquefaciens* on quality parameters of pears. Thus, it is in need of exploring the efficacy of *B. amyloliquefaciens* in control of gray mold decay and its effect on postharvest quality attributes of pears.

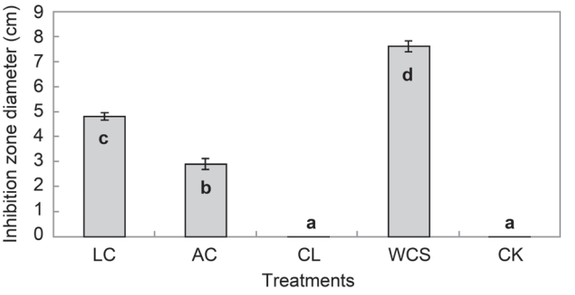

Fig. 1. Efficacy of different treatments in controlling of growth of *B. cinerea* on plates.

LC, liquid cultures; AC, autoclaved cultures; CL, Culture filtrates; WCS, 1×10^8 CFU/ml washed cell suspension; CK, sterile distilled water. Inhibition zone diameters were measured after 7 days at 28°C. Means are averaged values of three trials ± standard error. Bars represent standard errors. Values followed by different lowercase letters are significantly different at the 0.01 probability level according to analysis by Duncan's multiple range tests.

Antagonism *in vitro*. On synthetic potato medium plates, the inhibition zone diameter of washed cell suspension of BA3 was 7.6 cm (Fig. 1) and was significantly larger than that of control and other treatments. Liquid cultures and autoclaved cultures also significantly inhibited the development of *B. cinerea*. Inhibition zone diameters, respectively, were 4.8 cm and 2.9 cm. But those were significantly smaller than that of washed cell suspension. Culture filtrates and sterile distilled water did not have any inhibitory effect on *B. cinerea* so the inhibition zone diameters were 0 cm.

The inhibition zone diameters of washed cell suspension of BA3 were obviously larger than control and all the other treatments and it indicated the cell suspension was the best agent among what we have used against *B. cinerea* (Hu *et al*., 2015). So the cell suspension was utilized in the next experiments.

Efficacy of BA3 on conidial germination of *B. cinerea*. The spore germination of control was 62.88% after incubated at 28°C for 20 hours, and the germination of treatment was 18.72%. At the same time, the germ tube length of *B. cinerea* of control was 30.44 μm, which of treatment was 12.85 μm (Table I).

B. amyloliquefaciens significantly controlled spore germination and germ tube length of *B. cinerea.* Lutz *et al.* (2013) indicated that there are differences between different strains' inhibiting effect and different pathogens. The germination inhibition percentage of BA3 is higher than most yeast strains in Lutz *et al.* (2013) and Spadaro *et al.* (2013). Several biocontrol mechanisms have been suggested as being effective against postharvest fruit diseases (Jamalizadeh *et al*., 2011),

Table I
Efficacy of *B. amyloliquefaciens* strain BA3 on conidial germination of *B. cinerea*

Treatments	Spore germination (%)	Germ tube length (μm)
Control	62.88 ± 3.78a	30.44 ± 1.79a
B. amyloliquefaciens	18.72 ± 1.26b	12.85 ± 0.75b

Germination rate and germ tube length were measured after 20 h incubation at 28°C in PDB. Means are averaged values of three trials ± standard error. Values followed by different lowercase letters are significantly different at the 0.01 probability level according to analysis by Duncan's multiple range tests. The same as below.

competition for nutrients is the possible mechanism that BA3 inhibit the spore germination.

Population studies of BA3 on fruit wounds. At the start of the experiment (time 0), the population of *B. amyloliquefaciens* was 6×10^4 CFU/wound, and then it increased rapidly to 4.7×10^6 CFU/wound (Fig. 2).

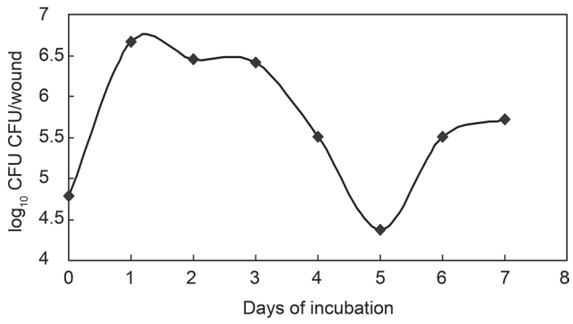

Fig. 2. Population dynamics of *B. amyloliquefaciens* on fruit wounds incubated at 25°C.
Data were pooled from three experiments and each point representing the mean colony counts from three replicate fruits.

In the following three days (1st day to 3rd day), the populations remained stability basically. But on the fourth day, there was a declining trend of. When on the fifth day, *B. amyloliquefaciens* population touched bottom of 2.4×10^4 CFU/wound, then increased to 4×10^5 CFU/wound and kept stable.

To select an antagonist suitable for postharvest application, it is necessary to look for what can be adapted for the environment of wounds and make use of nutrient sources, then grow it and proliferate it well (Manso and Nunes, 2011; Yu *et al*., 2013). The population of *B. amyloliquefaciens* strain BA3 increased rapidly and remained stability on the wounds of pears in the first three days which demonstrated that BA3 has the potential as an antagonist. But the population declined to 2.4×10^4 CFU/wound on the fourth day. The pathogens might attach to the wounds, begin to grow, and then compete for nutrition and space against *B. amyloliquefaciens.* Afterwards the antagonist became the dominant bacteria and the population of BA3 kept stable. These suggested that the competition for nutrient sources and space may be one of the mechanisms of action to inhibit the pathogen. This result is different from Zhang *et al*. (2008a) and Hu *et al*. (2015). It is probable that *B. amyloliquefaciens* needs a transitional period to control the pathogens.

Efficacy of BA3 in control of gray mold decay of pears. After incubation at 25°C for 7 days, the infection rate of control fruit was 100%. And at the concentration of *B. amyloliquefaciens* of 1×10^8 CFU/ml, it decreased to 53%, which was significantly lower than the control (Table II). The infection rate of the concentration of *B. amyloliquefaciens* at 1×10^8 CFU/ml was not significantly lower than 1×10^7 CFU/ml, but data indicated that the lesion diameters at 1×10^8 CFU/ml were significantly smaller than at 1×10^7 CFU/ml. Moreover, lesion diameter of the control was 4.80 cm, which of *B. amyloliquefaciens* at the concentration of 1×10^8 CFU/ml was significantly smaller than the control, being 0.91 cm. After cultivation for 30 days at 0°C, the infection rate of the control was 86.3%, while at concentration 1×10^8 CFU/ml it was significantly lower than the control, being 29.8%. Being different from cultivated at 25°C, infection rate at 1×10^8 CFU/ml was significantly lower than that at 1×10^7 CFU/ml. However, the lesion diameters of the concentration at 1×10^6 CFU/ml and 1×10^7 CFU/ml did not show significant differences. Both infection rate and lesion

Table II
Efficacy of *B. amyloliquefaciens* strain BA3 in control of gray mold decay of pears

Concentrations of *B. amyloliquefaciens* (CFU/ml)	25°C		0°C	
	Infection rate (%)	Lesion diameter (cm)	Infection rate (%)	Lesion diameter (cm)
0 (CK)	100 ± 0a	4.80 ± 0.06a	86.3 ± 1.26a	4.18 ± 0.19a
1×10^6	91.5 ± 3.38b	3.82 ± 0.11b	61.2 ± 1.68b	2.73 ± 0.08b
1×10^7	68.2 ± 4.44c	2.78 ± 0.27c	47.8 ± 1.40c	2.37 ± 0.09b
1×10^8	53.0 ± 5.38c	0.91 ± 0.14d	29.8 ± 1.30d	0.75 ± 0.11c

Infection rate and lesion diameter were recorded after storage at 25°C for 7 days or 0°C for 30 days.

Table III
Efficacy of *B. amyloliquefaciens* strain BA3 on postharvest quality parameters of pears

Treatments	Mass loss (%)	Ascorbic acid (mg/100g)	Titratable acidity (% malic acid)	Fruit firmness (N)	Total soluble solids (%)
Control	1.74 ± 0.49a	0.651 ± 0.056a	0.067 ± 0.0053a	11.96 ± 0.40a	11.11 ± 0.423a
Antagonist	1.25 ± 0.04a	0.881 ± 0.083b	0.078 ± 0.0070a	13.24 ± 0.40a	11.22 ± 0.324a

diameter of each concentration at 0°C were smaller than at 25°C.

The results reported in Table II show that, the concentration of BA3 significantly influenced the development of *B. cinerea* in pear. Hu *et al.* (2015) and Li *et al.* (2011) demonstrated that the higher the concentration of *B. amyloliquefaciens,* the lower the infection rate and the smaller the lesion diameter. Similarly, there is a direct correlation between the concentration of *B. amyloliquefaciens* strain BA3 and biocontrol effectiveness. When the concentration of *B. amyloliquefaciens* was 1×10^8 CFU/ml, the infection rate was reduced almost by one half, and the lesion diameters were contained within 1 cm.

Since most fruits are stored at low temperatures to extend shelf-life, being able to inhibit decay at low temperatures condition is the criterion for selecting an antagonist (Manso and Nunes, 2011). Stored at 0°C, the effect of BA3 in controlling of gray mold decay is better than at 25°C, suggesting that *B. amyloliquefaciens* had great potential in inhibiting gray mold decay of pears and could be utilized as a biological control agent at low temperatures.

According to studies conducted by Lutz *et al.* (2013), limiting spore germination of pathogens may be one of the major mechanisms of action of *B. amyloliquefaciens*. However, the interactions among host, pathogen, antagonist and microorganisms are complicated (Liu *et al.*, 2010), and the action mechanisms of antagonists against pathogens are not single (Arguelles-Arias *et al.*, 2009). This requires further research and more accurate descriptions.

Quality parameters of postharvest pears treated with BA3. According to Table III, the pears treated with *B. amyloliquefaciens* showed no significant differences with regard to mass loss, titratable acidity, firmness and total soluble solids compared with the control. But the ascorbic acid of pears treated with *B. amyloliquefaciens* was significantly higher than that of control fruits.

Not impairing the quality attributes of fruits is one of the conditions of an ideal biocontrol agent (Liu *et al.*, 2010). Moreover, the ascorbic acid of pears treated with *B. amyloliquefaciens* was significantly enhanced. This is different from other literature data (Özden and Bayindirli, 2002; Zhang *et al.*, 2008a; Luo *et al.*, 2015) which discussed the effect of antagonists on quality parameters of postharvest fruits. All of this further illustrates that *B. amyloliquefaciens* strain BA3 has a commercial potential.

In our experiments, *B. amyloliquefaciens* was isolated from the "douchi" and therefore is not harmful to human health. All of the results from the study indicate that *B. amyloliquefaciens* strain BA3 has the potential to be used as a biological control agent.

Our experiments did not fully assess the effect of *B. amyloliquefaciens* in control of gray mold decay of fruits, considering storage conditions or the mixture of other materials that improve the biocontrol efficacy so as to explore better means of biocontrol. We should also focus our further studies on the commercial application of *B. amyloliquefaciens.*

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