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EFFECTS OF CHITOSAN COMBINED WITH MICROBES ON BLUE MOLD OF APPLES

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Abstract

Investigation on the inhibitory effect of chitosan combined with antagonistic bacteria on pathogenic fungi, at different concentrations on *Penicillium expansum* were studied *in vitro* and *in vivo* with Japanese Red Fuji apples. The chitosan solution with the concentration of 2% had the strongest resistance to *Penicillium expansum* and blue mold decay without affecting the growth of *Bacillus amyloliquefaciens* BA-16-8. Both the bacterial suspension and cell-free fermentation liquor of *Bacillus amyloliquefaciens* BA-16-8 could inhibit *Penicillium expansum*, and the former is superior to the latter in effect. *Bacillus amyloliquefaciens* BA-16-8 combined with chitosan can inhibit the activity and toxin-producing ability of *Penicillium expansum*.

Introduction

China is a big country in fruit planting and export, among which apple yield ranks first in the world. However, the infection of postharvest diseases causes fruits to rot seriously, which brings huge economic losses to apple planting and its by-product processing industry (Leng *et al.* 2023, Fu *et al.* 2022a). Among them, Blue mold is one of the most important diseases that causing rotting of apples after harvest. And effective control of this disease has become a problem demanding prompt solution. It is shown by relevant researches that *Penicillium expansum* is one of the most important pathogenic fungi causing blue mold decay of apples, which is widely distributed in apples and their seeds, and can not only cause apples to rot from the outside to the inside, but also secrete patulin in the process of invasion (Mahunu *et al.* 2018, Yu *et al.* 2020, Fu *et al.* 2022b). With regard to the problem of apple rotting after harvest, at present, chemical fungicides are mostly used to inhibit *Penicillium expansum* and other pathogenic fungi to reduce the rotting rate of apples (Wang *et al.* 2019, Riachy *et al.* 2021). However, while killing or inhibiting bacteria, these chemical fungicides will lead to a series of problems such as food safety risks and environmental pollution (Ge *et al.* 2018, López *et al.* 2021, Wang *et al.* 2021).

Penicillium expansum and *Bacillus amyloliquefaciens* BA-16-8 (Fu *et al.* 2020a) were selected and cultured in the early stage of the study, which were turned to be suitable for antiseptic preservation of fruits through bacteriostatic performance and safety test (Fu *et al.* 2020b). And then, in order to solve the problem of reduced effectiveness in prevention and control caused by poor adhesion of its bacterial suspension, chitosan was selected as filmogen to assist the bacterial suspension in adhesion onto surface of apples.

Chitosan is a product formed after deacetylation of chitin with film-forming property (Assaf *et al.* 2020). Studies have shown that chitosan, when coated on postharvest fruits, can not only effectively block the invasion of pathogenic fungi through mechanical barrier, but also induce

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fruit resistance by its hydrolysates (Wang *et al.* 2023). On this basis, this study mainly discusses the inhibitory effect of chitosan on pathogenic fungi and its influence on antagonistic bacteria. And it also gives a comparison of the control effect on pathogenic fungi of chitosan alone, antagonistic bacteria alone, and their compound for the purpose of providing theoretical basis for application of *Bacillus amyloliquefaciens* BA-16-8 in postharvest corrosion prevention for apples.

Materials and Methods

Bacillus amyloliquefaciens BA-16-8 was isolated, cultured and identified by Bioengineering Laboratory of Shaanxi Normal University, *Penicillium expansum* was donated by Shaanxi Institute of Microbiology, Chinese Academy of Sciences. The reagents used in the experiment were chitosan, pectinase, patulin and fengycin.

The red Fuji apples were purchased from a well-managed orchard in Luochuan County, Shaanxi Province. The apples were even in size, consistent in maturity and free from mechanical damage and disease, and transported back to the laboratory immediately after harvest. The apples were cleaned with tap water, and then disinfected with 0.1% sodium hypochlorite for 1 min.

The cell-free fermentation solution of antagonistic bacteria *B. amyloliquefaciens* BA-16-8 was prepared according to Xie (2016).

According to Baloh (2022), *Penicillium expansum* spore suspension was prepared and spore germination was made.

The apples were drilled and added with different concentrations of *Bacillus amylolyticus* suspension and chitosan solution respectively, and kept at constant temperature at 30℃. Samples were collected at 72, 96 and 120 hrs, respectively. The diameter of lesions was measured and the incidence was calculated.

Software SPSS19.0 was used for data analysis.

Results and Discussion

The colony formation and spore germination were observed after culture of *Penicillium expansum* spore suspension on the plate was coated with chitosan solution at different concentrations. Results showed that, spore germination was completely inhibited by chitosan solution, and the inhibition effect would be strengthened when the concentration of chitosan solution was increased (Fig. 1). It reached the peak effect when the concentration of chitosan was at 2%. In addition, it was found out that, it was not the case that the higher concentration of the chitosan is, the stronger the inhibition effect would be triggered.

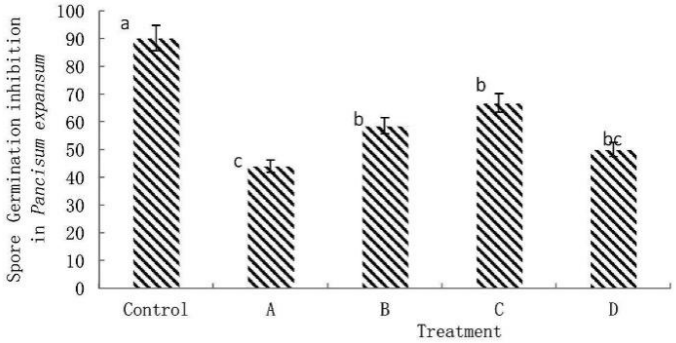

Fig. 1. Germination of conidia of *Penicillium expansum* under different concentrations of chitosan liquid.

The colony formation and spore germination were observed after culture of *Penicillium expansum* spore suspension on the plate was coated with *Bacillus amyloliquefaciens* BA-16-8 suspension at different concentrations and cell-free fermentation liquor. Results showed that, spore germination was completely inhibited by *Bacillus amyloliquefaciens* BA-16-8 suspension, and the inhibition effect was positively correlated with the concentration of bacterial suspension (Fig. 2). Based on the experiment, *Bacillus amyloliquefaciens* BA-16-8 suspension at the concentration of 108/ml was selected for further study.

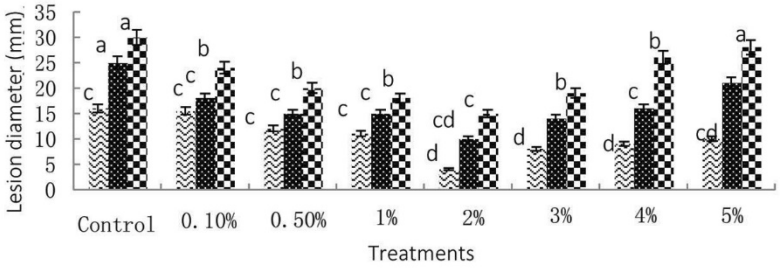

Fig. 2. Germination of conidia of *Penicillium expansum* under different concentration of *Bacillus amyloliquefaciens* BA-16-8 fermentations broth and cell-free fermentation broth.

Chitosan at different concentrations was used in treatment of apples, and the effect on blue mold decay of apples caused by different treatments was observed. After 72 hrs, the inhibitory effect is as shown in Fig. 3. It can be seen that, Chitosan has remarkable effect on control of apple blue mold decay, and can significantly inhibit the spread of the lesion. And within certain range, the inhibitory effect is stronger as the increase in concentration of chitosan solution, which is reflected by the smaller diameter of the lesion. Nevertheless, higher concentration is not always the better. It was found not in the experiment that, the lesion in apples became bigger again when the concentration was 5%. In general, it is the best to use chitosan solution at the concentration of 2% as treating liquid.

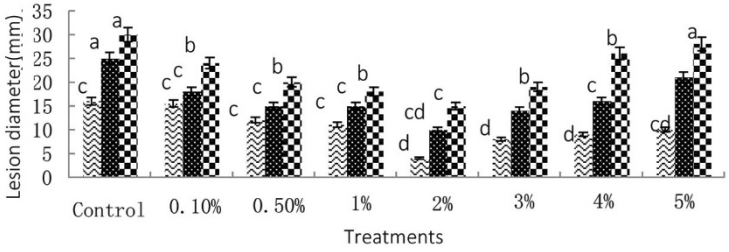

Fig. 3. The lesion diameter of blue mold owing to *Penicillium expansum* in apple under different concentrations of chitosan liquid.

Figure 4A and 4B, both the antagonistic bacteria *Bacillus amyloliquefaciens* BA-16-8 suspension and its cell-free fermentation liquor play a positive role in postharvest blue mold decay caused by *Penicillium expansum*, and lesion diameter and rotting rate of the apples inoculated with antagonistic bacterial suspension and fermentation liquor are significantly lower than these of the control group. In addition, the control effect becomes greater with the increase in concentration of the antagonistic suspension, which is shown by the fact that, lesion and rotting rate decline with the increase in concentration of the antagonistic suspension. What's more, the antagonistic

suspension is superior cell-free fermentation liquor in its efficacy with extension of time. It is assumed that, the antimicrobial substance, in the fermentation liquor, fengycin, for instance, declines in its content while playing the role of bactericidal effect by binding to mycelial membrane or DNA in *Penicillium expansum*. Whereas, the strain cells in the bacterial suspension can still produce fengycin through metabolism. And bacterial cells themselves can also act as an *Penicillium expansum* suppressor through space competition or nutritional competition.

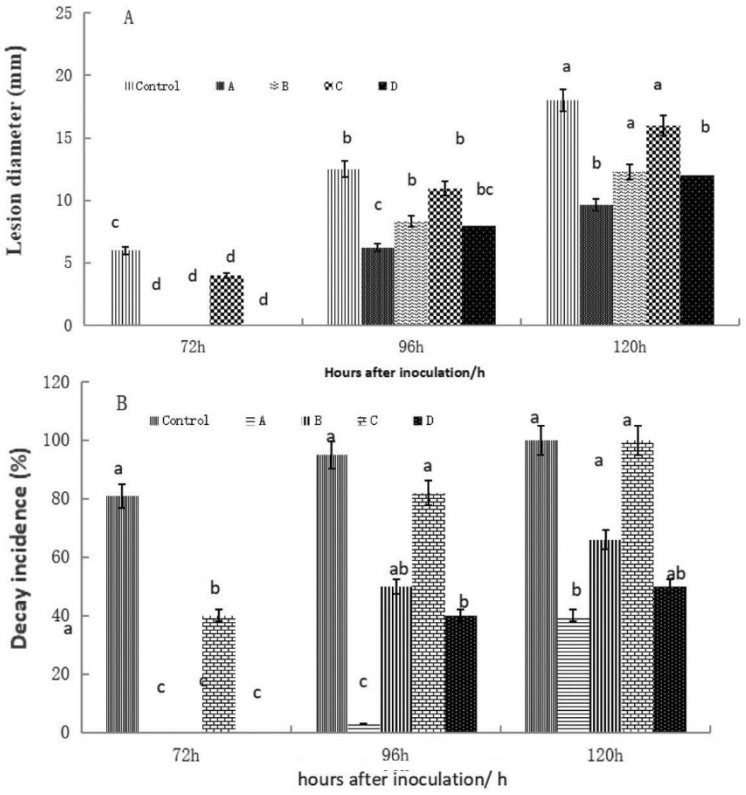

Fig. 4. Lesion diameter (A) and decay incidence (B) of blue gray caused by *Penicillium expansum* in apple under different treatments.

The influence imposed on growth of *B. amyloliquefaciens* BA-16-8 by chitosan solution at different concentrations is as shown in Fig. 5. When the concentration of chitosan solution is equal to or greater than 3% (w/v), growth of *Bacillus amyloliquefaciens* is significantly inhibited ($P < 0.05$); when it concentration is less than 3%(w/v), the amount of *Bacillus amyloliquefaciens* falls by 2.5 order of magnitudes compared to the control group. In order to study the inhibitory effect on blue mold decay and the influence on *Bacillus amyloliquefaciens*, chitosan solution at the concentration of 2% (w/v) is to be used in subsequent studies.

Penicillium expansum under the combined treatment of *Bacillus amyloliquefaciens* BA-16-8 suspension and 2% chitosan solution was used for infection of apple fruits in vitro, the results are as shown in Fig. 6. It turns out that combined treatment of bacterial suspension of *Bacillus amyloliquefaciens* BA-16-8 and 2% chitosan solution plays a synergistic effect in control of blue

mold decay. In addition, consistent with the results in Fig. 4, the antiseptic effect of *Bacillus amyloliquefaciens* suspension and chitosan composite is better than that of cell-free fermentation liquor and chitosan composite.

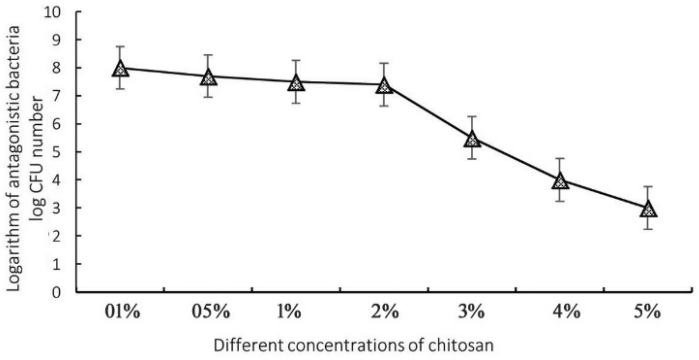

Fig. 5. Effect of chitosans at different concentration on growth of *Bacillus amyloliquefaciens*.

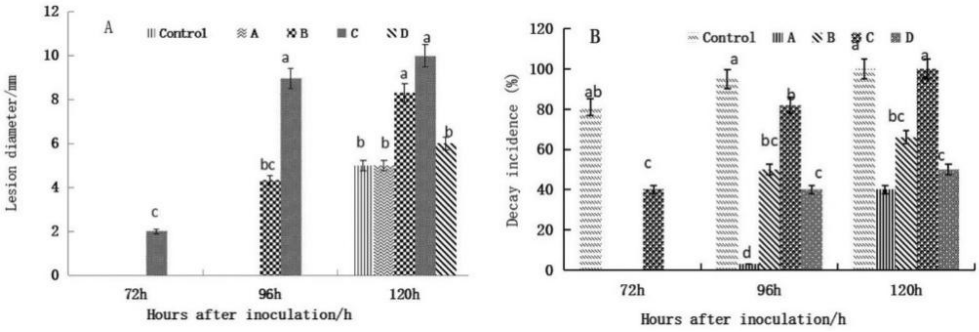

Fig. 6. Lesion diameter (A) and decay incidence (B) of blue gray caused by *Penicillium expansum* in apple under different treatments.

Film preservation means that edible film or coating is wrapped on the surface of fruits and vegetables by dipping or spraying, and the protective layer attached to the outside can effectively increase the content of CO_2 inside the fruit and inhibit fruit respiration. It blocks water evaporation and effectively inhibits the growth of microorganisms, thereby reducing the loss of nutrients in fruits and vegetables and prolonging the storage and freshness life (DeGenring *et al.* 2023).In addition, the coating can incorporate some beneficial substances such as antioxidants, antimicrobials and flavorings etc. are transferred to food. Apple coating materials generally include polysaccharides, proteins and lipids, and plasticizers such as glycerol, sorbitol and propylene glycol can also be added to the material matrix to enhance the function (Godana *et al.* 2020) .Due to the advantages of non-pollution, edible, long shelf life and improved nutritional quality of fruits and vegetables, film preservation has been widely used in postharvest preservation technology of fruits and vegetables (Li *et al.* 2015). Different coatings had different effects on the quality of fresh apples after harvest.

Chitosan is a biopolymer derived from chitin and has been widely studied for its antimicrobial properties and biodegradability.The antibacterial mechanism of Chitosan lies in its positive

charge,which may compete with Ca^{2+} for negatively charged bacterial membranes (Stocco *et al.* 2019) .However, the comprehensive performance of a single Chitosan membrane is not good, and people usually add antibacterial biopolymer or antibacterial agent to prepare Chitosan-composite membranes which can be used to control microorganisms (Wang *et al.* 2019).

In the present study, the inhibitory effect on *Penicillium expansum* of chitosan solution at different concentrations *in vitro* and in fruit is compared firstly, and it is thereby concluded that chitosan solution at 2% concentration had the strongest resistance to *Penicillium expansum* and blue mold decay. And then, the bacterial suspension and cell-free fermentation liquor of *B. amyloliquefaciens* BA-16-8 is separately used in biocontrol experiments on blue mold decay of apples so as to detect its resistance to disease and bring about reflection on the inhibitory effect on secretion of patulin by *Penicillium expansum*. It turns out that, patulin can be detected in both the control group and the experimental group on the third day, and the content gradually rises as the increase of the inoculation time. Nevertheless, patulin secreted by the *Penicillium expansum* treated by antagonist bacterial is far less than that for the control group. Thus, it can be inferred that the antagonistic strain may inhibit toxin secretion by restraining the growth of *Penicillium expansum*, or it might be that the antagonistic strain can degrade patulin. In addition, toxin secreted by the *Penicillium expansum* treated by cell-free fermentation liquor of antagonistic bacteria is far less than that of the control group. However, the toxin content increases gradually with the time prolongs, which indicates that cell-free fermentation liquor of antagonistic bacteria inhibits its toxin-producing ability of *Penicillium expansum* by suppressing its activity. Based on the previous studies, we have proved that the main active bacteriostatic constituent in cell-free fermentation liquor of *B. amyloliquefaciens* BA-16-8 is fengycin. When studying its mechanism of action, we find out that fengycin could suppress the activity of Phospholipase A in *Penicillium expansum*, which is responsible for expression of Oxylipins and other substanceand the latter is the main regulatory factor controlling the synthesis of biological products. On this ground, fengycin is assumed to have certain influence on production of patulin, which needs to be verified by further experiments. In recent years, more and more antagonistic bacteria have been developed and applied in postharvest biocontrol for fruits and vegetables. However, there have been few studies on the role of antibiotic treatment in toxin secretion by bacteria. Through this experiment, it was found find that although antagonistic bacteria could inhibit growth of pathogenic fungi, and suppress its secretion of toxins, suggesting to take into consideration the content of toxin secreted by antagonistic bacteria in the process of biocontrol and include such influence into evaluation of biocontrol agents.

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