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Integrated control of blue mold in pear fruit by combined application of chitosan, a biocontrol yeast and calcium chloride

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ABSTRACT

This study evaluated the performance of chitosan at different concentrations alone, and in combination with a biocontrol yeast *Cryptococcus laurentii* and calcium chloride, on the reduction of the blue mold decay caused by *Penicillium expansum* in pears. The efficacy of chitosan in inhibiting fungal infections in pear wounds was decreased as incubation time increased. The combination of chitosan at 0.5% and *C. laurentii* resulted in more effective mold control than chitosan or *C. laurentii* alone, although chitosan at 0.5% inhibited growth of this biocontrol yeast *in vitro* and *in vivo*. The effectiveness of the combined treatment with chitosan and biocontrol yeast was also significantly reduced with the incubation time. Calcium chloride had little antifungal activity, however integration of calcium chloride with chitosan and *C. laurentii* resulted in a more effective and stable reduction in the fungal decay compared with the treatment with either chitosan or with *C. laurentii* alone.

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1. Introduction

Penicillium expansum link is the most important fungal pathogen of pear fruit, which is responsible for substantial global economic losses (Rosenberger, 1990). Although the use of synthetic chemical fungicides remains the major method for controlling postharvest decay, there is an urgent demand to develop alternative approaches to reduce or even replace use of fungicides because of concerns regarding the potential impact on human health and the environment by fungicide residues (Janisiewicz and Korsten, 2002; Droby et al., 2009).

Chitosan, an N-acetylated derivative of the polysaccharide chitin, holds great potential for food applications with its unique physiological and biological properties (Bautista-Baños et al., 2006; Dutta et al., 2009). Postharvest treatments of fruit and vegetables with chitosan are reported to create a semi-permeable film that delays fruit ripening and prolongs shelf-life (Bautista-Baños et al., 2006; Ali et al., 2011) and inhibits fungal decay by direct antifungal activity (Bautista-Baños et al., 2006; Romanazzi et al., 2009; Meng et al., 2010b; Reglinski et al., 2010), or by induction of host resistance to pathogens (Fajardo et al., 1998; de Capdeville et al., 2002; Zeng et al., 2010).

Biological control with antagonistic microorganisms has been considered the most promising alternative method for inhibition of postharvest diseases in recent years, especially for control of wound-invading pathogens (Janisiewicz and Korsten, 2002; Janisiewicz and Conway, 2010). At present there are several commercial products available in the market for postharvest use, such as Biosave (*Pseudomonas syringae*) registered in the USA and "Shemer" (*Metschnikowia fructicola*) registered in Israel (Droby et al., 2009).

Calcium chloride ($CaCl_2$) is widely used as a preservative and firming agent in the fruit and vegetable industry (Martín-Diana et al., 2007). The mechanisms by which $CaCl_2$ reduces diseases of harvested fruit include induction of host resistance, direct inhibitory effects, and complex interactions taking place between calcium ions, the host, and the pathogen (Bastiaanse et al., 2010; Janisiewicz and Conway, 2010).

Unfortunately, none of the non-fungicidal methods, when used alone, can provide satisfactory levels of decay control compared with synthetic fungicides. Therefore, from a practical viewpoint, a combination of different methods is needed for development of an effective alternative approach (Janisiewicz and Korsten, 2002; Droby et al., 2009; Walters, 2009; Jijakli, 2011).

It has been reported that the biocontrol efficacy of *Cryptococcus laurentii* can be improved by chitosan in reducing *P. expansum* infections in apple fruit wounds (Yu et al., 2007). Pre-harvest treatment with *C. laurentii* and chitosan (Meng and Tian, 2009), or pre-harvest spraying by *C. laurentii* and followed by postharvest treatment with chitosan (Meng et al., 2010a), significantly reduced natural decay of grape fruit. On the other hand, it has been found that co-treatment with *C. laurentii* and $CaCl_2$ resulted in a significant reduction of blue

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mold in apple (Lima et al., 2005) and gray mold in pear (Zhang et al., 2005). In addition, enhanced effectiveness was observed by integrated applications of glycochitosan (0.2%) with biocontrol yeast *Candida saitoana* against postharvest blue and gray molds of apple as well as green mold of oranges and lemons (El-Ghaouth et al., 2000a,b; de Capdeville et al., 2002). Recently, Rahman et al. (2009) reported that a combination of the antagonistic bacteria *Burkholderia cepacia* with chitosan (0.75%) and $CaCl_2$ was effective in controlling anthracnose in papaya caused by *Colletotrichum gloeosporioides*.

However, to the best of our knowledge, there was no information concerning with the effect of combination of chitosan and *C. laurentii* on reduction of postharvest decay of pear fruit. Therefore, this study was conducted to investigate the potential of chitosan alone or in combination with *C. laurentii* on control of blue mold in pear fruit, and the effect of the addition of $CaCl_2$.

2. Materials and methods

2.1. Chemicals, fruit and microorganisms

Crab-shell chitosan (deacetylation of about 90%) with the intrinsic viscosity of 12 centipoises (cps) and calcium chloride were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Chitosan was dissolved in 0.1% (v/v) acetic acid and subsequently adjusted to pH 5.0 and then diluted with sterile distilled water as required.

Asian pear (*Pyrus pyrifolia* Nakai, cultivar "Shuijing") fruit were obtained from a commercial source at Hangzhou, Zhejiang province, China. After transportation to the laboratory, the samples were selected for uniformity of size, ripeness and any fruit with apparent injuries or decay were removed. Prior to inoculation, fruit were sterilized by drenching in a water solution of 0.1% sodium hypochlorite for 1 min and then washed thoroughly with tap water, and air-dried at room temperature (20 °C).

The antagonist *C. laurentii* was originally isolated from the surfaces of pear fruit (Zhang et al., 2005) and was cultured in 50 mL of nutrient yeast dextrose broth (NYDB, containing 8 g nutrient broth, 5 g yeast extract, and 10 g glucose in 1 L of distilled water) in 250 mL flasks at 28 °C for 24 h on a rotary shaker at 200 rpm. The yeast cells were harvested by centrifuging at $7000 \times g$ for 10 min and were washed twice with sterile distilled water to remove the growth medium. The cells were counted using a hemocytometer and then were diluted with sterile distilled water as required.

The pathogen *P. expansum* was isolated from pear fruit and cultured on potato-dextrose agar (PDA) medium (containing an extract from 200 g potatoes, 20 g glucose and 20 g agar in 1 L of distilled water) at 26 °C in the dark. Spore suspensions were prepared by flooding 7-day-old sporulating cultures of *P. expansum* with sterile distilled water. Spore concentration was calculated by a hemocytometer and then diluted with sterile distilled water as required.

2.2. Effect of chitosan on populations of antagonist yeast in vitro

Yeast cells (about 500 cells mL^{-1}) were mixed with various chitosan concentrations at 0, 0.001, 0.01, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0% (w/v) in a final volume of 5 mL and were kept for 1 min. Then 100 μL of the suspensions were plated on NYDA medium (containing 8 g nutrient broth, 5 g yeast extract, 10 g glucose and 20 g agar in 1 L of distilled water). After 48 h incubation at 28 °C, the colonies per plate were counted. Data were expressed as the percentage of cells survival of the control (%). Each treatment was replicated three times with four plates per replicate, and the experiment was conducted twice with similar results.

2.3. Effect of chitosan on population growth of antagonist yeast in vivo

The fruit were wounded (5 mm diameter by 3 mm deep) using a sterile borer. There were four wounds for each fruit. Then the wounds were treated with 50 μL of a cell suspension of *C. laurentii* at 1×10^7 cells mL^{-1} containing chitosan at various concentrations of 0, 0.1, 0.5 and 1.0% (w/v), respectively. After treatments, the samples were stored in enclosed plastic trays to maintain a high relative humidity at 20 °C and tissue was removed with a sterile cork borer (1 cm diameter by 1 cm deep) and ground with a mortar and pestle in 10 mL of sterile distilled water every 24 h after treatment. The cells were counted using a hemocytometer. There were four replicates per treatment with six fruit samples per replicate, and the experiment was conducted twice with similar results.

2.4. Efficacy of chitosan, antagonist yeast and calcium chloride in control of blue mold in pear fruit wounds

Fruit were wounded as described above and then treated with 50 μL of one of the following: (1) chitosan at concentrations of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 1% (w/v), respectively; (2) cell suspensions of *C. laurentii* alone at 1×10^8 cells mL^{-1} or with chitosan at the concentrations of 0.1, 0.5 and 1% (w/v), respectively; (3) $CaCl_2$ at 1% (w/v) alone or with *C. laurentii* at 1×10^8 cells mL^{-1} or with the combination of *C. laurentii* at 1×10^8 cells mL^{-1} and chitosan at 0.5% (w/v), respectively; (4) sterile water as the control. Two hour later, 20 μL of *P. expansum* spores at 1×10^4 spores/mL were inoculated into each wound. After 4 h, the samples were stored in enclosed plastic trays to maintain a high relative humidity at 20 °C. The numbers of the infected fruit wounds and their lesion diameters were examined daily after inoculation. The percentage inhibition (%) for each treatment was calculated by comparison with the water control:

$$\text{Inhibition (\%)} = \left[\frac{\text{the average lesion diameter of infected wounds in the control} - \text{the average lesion diameter of infected wounds in the treatment}}{\text{the average lesion diameter of infected wounds in the control}}\right] \times 100.$$

There were four replicates of 36 wounds per replicate and the experiment was conducted twice with similar results.

2.5. Statistical analyses

All treatments were arranged in a randomized complete block design and conducted at least twice. The data are from one individual experiment and are representative of two independent experiments with similar results. The data were subjected to analysis of the variance (ANOVA) in the Statistical Program (SPSS/PC ver. II.x, SPSS Inc. Chicago, IL, USA). The statistical significance was applied at the level $P = 0.05$. When the analysis was statistically significant, Duncan's multiple range test was applied to separate means.

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