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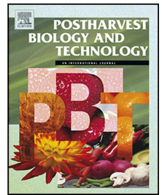

Colloidal chitin reduces disease incidence of wounded pear fruit inoculated by *Penicillium expansum*

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ABSTRACT

The objective of this study was to determine the effect of colloidal chitin on the control of blue mold caused by *Penicillium expansum* in pear fruit. The results indicated that colloidal chitin at 0.1–1% reduced disease incidence of blue mold when *P. expansum* was inoculated 24 h after colloidal chitin treatment. Moreover, the co-treatment with colloidal chitin and *P. expansum* induced a significant increase in the activities of polyphenol oxidase and peroxidase, compared with those that were treated with colloidal chitin or inoculated with the pathogen alone. In addition, colloidal chitin had no adverse effect on pear fruit quality. These results suggest that the application of colloidal chitin may be an effective method to control postharvest fungal diseases, and its mechanisms of action may be associated with the elicitation of defense-related enzymes in fruit.

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1. Introduction

Blue mold caused by *Penicillium expansum* is one of the major postharvest diseases of pear fruit that causes considerable economic loss worldwide (Yu et al., 2012). On the other hand, the overuse of synthetic fungicides has attracted extensive attention about the environment and human health, and persistent use has led to increasing resistance of pathogens. Therefore, it is imperative to find an eco-friendly alternative to control the fungal decay (Droby et al., 2009; Sharma et al., 2009).

Induced resistance by using physical, biological, and/or chemical elicitors is one of the promising approaches to replace synthetic fungicides and has received increasing attention over recent years (Sanzani et al., 2010). The advantages of disease control by elicitors are that they are nontoxic, biodegradable and offer a long-lasting and systemic resistance to a broad spectrum of pathogens (Lu et al., 2014a,b). Several chemical elicitors have been reported to induce resistance of fruit to fungal pathogens, such as salicylic acid (Cao et al., 2013), γ-aminobutyric acid (Yu et al., 2014) and methyl jasmonate (Yu et al., 2009).

Chitin, as the second most abundant biopolymer, has various applications in agriculture and the food industry (Prashanth and Tharanathan, 2007), for instance, as a biopesticide approved by the US Environmental Protection Agency (EPA) and as a food additive approved by the US Food and Drug Administration (FDA). In the last decade, many reports showed that chitin could enhance the efficacy of biocontrol agents like *Rhodotorula glutinis* (Ge et al., 2010), *Rhodosporidium paludigenum* (Lu et al., 2014a,b), *Pseudomonas fluorescens* and *Bacillus subtilis* (Vivekananthan et al., 2004), to control postharvest diseases of fruit. However, reports regarding the effect of colloidal chitin on control of postharvest fungal diseases are yet to be demonstrated.

The objectives of this study were to assess the effect of colloidal chitin on the control of blue mold caused by *P. expansum* in pear fruit, as well as to evaluate antifungal activity of colloidal chitin on *P. expansum in vitro* and *in vivo*, and elicitation of polyphenol oxidase (PPO) and peroxidase (POD) by colloidal chitin and/or *P. expansum* in fruit.

2. Materials and methods

2.1. Fruit, pathogen and colloidal chitin

Pears (*Pyrus pyrifolia* Nakai. cultivar "Shuijing") were harvested at commercial maturity and selected based on uniform size, ripeness and absence of mechanical damage. After being disinfected by immersing in 0.1% (v/v) sodium hypochlorite solution for 1 min, the pears were washed with tap water and air dried at 25 °C.

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P. expansum was isolated from decayed pear fruit and maintained on potato dextrose agar (PDA), containing the extract from 200 g of potatoes (boiled for 10 min and filtered through eight layers of gauze), 20 g of glucose and 20 g of agar in 1 L of distilled water) at 25 °C for 7 days. Spore suspensions were obtained by flooding the sporulating cultures of *P. expansum* with sterile distilled water. The spore concentrations were determined using a hemacytometer and adjusted with sterile distilled water as required.

Colloidal chitin was prepared by chitin powder. Five grams of powdered chitin was suspended in 100 mL of 37% HCl and stirred on a gyratory shaker at 40 °C for 2 h. Then, 1 L of 95% ethyl alcohol was added. The pellet was collected by centrifugation at $3000 \times g$ for 20 min and washed with distilled water until the pH of the filtrate reached 6.5.

2.2. Effect of colloidal chitin on control of blue mold caused by P. expansum *in pear fruit*

Six wounds were made (5 mm diameter and 3 mm deep) on each pear fruit with a sterile borer. Each wound was treated with 30 μL of colloidal chitin at 0.01%, 0.1%, 0.2%, 0.5% and 1% (w/v), respectively. Wounds treated with sterile distilled water served as the control. After 24 h, each wound was inoculated with 30 μL of *P. expansum* spore suspensions. The pears were then air dried and incubated in trays covered with plastic to maintain 90% relative humidity (RH) in the dark at 25 °C. Each treatment included three replicates and each replicate consisted of nine pears. Each test was performed at least twice.

2.2.1. Effect of colloidal chitin at different treatment time on control of blue mold

Eight wounds were made on each pear fruit as above and each wound was treated with 30 μL of colloidal chitin at 0.1% or sterile distilled water as a control. After 0, 12, 24 and 36 h, fruits were inoculated with 30 μL of *P. expansum* spore suspensions in each wound and then air dried and stored as above. Each treatment included three replicates and each replicate consisted of nine pears. Each test was performed at least twice.

2.2.2. Effect of colloidal chitin on control of blue mold at low temperature

Pears were wounded as above and treated with 30 μL of colloidal chitin at 0.1% or sterile distilled water as a control. After 24 h, each wound was inoculated with 30 μL of *P. expansum* spore suspensions. The pears were then air dried and stored as above but at 4 °C. Each treatment included three replicates and each replicate consisted of nine pears. Each test was performed at least twice.

In above three tests, the concentration of spore suspensions was 1×10^4 spores mL^{-1}. The percentage of infected wounds was examined daily after inoculation.

2.3. Effect of colloidal chitin on growth and spore germination of P. expansum in vitro

Equal amount of *P. expansum* spores were mixed with colloidal chitin in final concentrations at 0.001%, 0.01%, 0.1%, 0.2%, 0.5% and 1% (w/v), and kept for 1 min. Then 100 μL of spore suspensions were spread on PDA plates. Colonies were counted after incubation at 25 °C for 2 days and the data were expressed as the CFU/plate. Each treatment included three replicates and each replicate consisted of six plates.

Colloidal chitin was added in potato dextrose broth (PDB, containing the extract from 200 g of potatoes and 20 g of glucose in 1 L of distilled water) in final concentrations at 0.001%, 0.01%, 0.1%, 0.2%, 0.5% and 1%, then equal amount of *P. expansum* spores were added to make a final concentration at 1×10^6 spores mL^{-1}. The mixtures were incubated on a rotary shaker at 3.33 s^{-1} at 28 °C. After 12 h, 150–200 spores per replicate were observed microscopically for germination rate. Spores were considered germinated when the germ tube length was equal to or greater than spore length. Each treatment included three replicates.

2.4. Effect of colloidal chitin on spore germination of P. expansum *in pear fruit wounds*

Wounds were made on each pear as above and each wound was treated with 30 μL of colloidal chitin at 0.1% or sterile distilled water as a control. After 0 or 24 h, each wound was inoculated with 30 μL of *P. expansum* spore suspensions at 1×10^7 spores mL^{-1}. Fruit were air dried and incubated in trays covered with plastic to maintain 90% RH in the dark at 25 °C. Then after 12 h, 150–200 spores per replicate were observed microscopically for germination rate. Each treatment included three replicates and each replicate consisted of three wounds.

2.5. Assay of enzyme activities of PPO and POD in pear fruit

The wounds were made as above and treated with (1) 30 μL sterile distilled water as the control; (2) 30 μL colloidal chitin at 0.1%; (3) 30 μL of the *P. expansum* suspensions at 1×10^4 spores mL^{-1}; (4) 30 μL of the *P. expansum* suspensions at 1×10^4 spores mL^{-1} 24 h after the treatment of colloidal chitin at 0.1%. Tissue samples surrounding the wounds (not decayed) of fruit were collected at the same intervals (0, 12, 24, 36 and 48 h after the treatment) and immediately frozen in liquid N_2, then stored at −80 °C for further analysis of enzyme activities.

Frozen tissue samples (1.0 g) were ground in a mortar and pestle with 10 mL of cold (4 °C) 50 mmol L^{-1} sodium phosphate buffer (pH 7.8) containing 1.33 mmol L^{-1} EDTA and 1% (w/v) polyvinylpyrrolidone (PVP). The supernatants were obtained by centrifuging at 4 °C for 15 min at $9000 \times g$ and used to measure enzyme activities and protein contents. The protein content was determined according to the method of Bradford (1976) using bovine serum albumin as a standard. There were three replicates per treatment with three samples per replicate.

Polyphenol oxidase (PPO) activity was determined by monitoring the rate of the increase in absorbance at 398 nm following the method of Mohammadi and Kazemi (2002). The reaction mixture contained 250 μL of 50 mmol L^{-1} sodium phosphate buffer (pH 6.4) containing 10 mmol L^{-1} pyrocatechol and 50 μL of crude enzyme. The reaction was allowed to proceed for 5 min by measuring at 398 nm every 30 s. One unit of PPO activity is defined as the increase of 0.01 in A_{398} in 1 min and the activity was expressed as U kg^{-1}.

Peroxidase (POD) activity was determined using the method of Lurie et al. (1997). The reaction mixture consisted of 140 μL of 50 mmol L^{-1} PBS (pH 6.4) containing 0.3% (v/v) guaiacol, 60 μL of 0.3% H_2O_2 and 100 μL of crude enzyme extract. The increase in absorbance at 470 nm was measured after H_2O_2 was added. One unit of POD activity was defined as the increase of 0.01 in A_{470} in 1 min and the activity was expressed as U kg^{-1}.

2.6. Fruit quality assay

The pears were immersed in colloidal chitin at 0.1%, 0.2% and 0.5% for 10 min and then air-dried. Pears treated with sterile distilled water served as the control. Samples were collected after 7 days of storage at 25 °C for quality evaluation. Total soluble solids (TSS) content was determined by measuring the refractive index of pear juice with a hand-hold refractometer (WZ-103, Top instrument Co., Ltd., China). Titratable acidity (TA) content was measured by titrating a 10 mL of filtered pear juice to pH 8.2 with 0.1 mol L^{-1}

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