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Effects of chitosan on control of postharvest blue mold decay of apple fruit and the possible mechanisms involved

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ABSTRACT

The effectiveness of chitosan in controlling blue mold decay of apple fruit caused by *Penicillium expansum* and the possible mechanisms involved were investigated in the present study. The application of chitosan significantly reduced the blue mold decay of the postharvest apple fruit and patulin accumulation, and no negative effect of chitosan treatment on fruit quality was observed after 15 days of storage. Changes of protein expression profiles of apple fruit upon chitosan treatment were analyzed by two-dimensional electrophoresis and 20 differentially expressed proteins were identified by mass spectrometry analysis. Several proteins involved in defense, carbohydrate catabolism and protein biosynthesis were up-regulated by chitosan treatment. Activated defense response and increased energy supply in chitosan treated fruit may constitute the molecular basis for increased resistance to the possible future pathogen infection. This study contributes to a better understanding of the cellular events in apple fruit under chitosan treatment in view of a promising alternative in controlling postharvest disease.

1. Introduction

Blue mold decay, caused by species of *Penicillium expansum* Link, is one of the most important postharvest diseases of apples and pears worldwide. Besides causing economic losses, *P. expansum* has also potential public health significance since it produces patulin (Andersen et al., 2004). This mycotoxin is toxic for animals and human, causing intestinal injuries, including epithelial cell degeneration, inflammation and hemorrhages (Speijers and Franken, 1988), and other studies have also demonstrated that patulin can induce oxidative damage in human cells, resulting in mutagenesis and carcinogensis (Liu et al., 2003). Although synthetic fungicides are the primary means to control postharvest diseases, growing concern for human and environmental health risks associated with pesticide usage, the development of fungicide-resistant strains, and the lack of approval of some of the most effective fungicides have created interest in exploring for alternative approaches for the disease management. Among these new control strategies, the use of plant or animal products with fungicidal activity and the application of antagonistic microorganisms, either alone or as part of an integrated pest management policy, can be considered (Spadaro and Gullino, 2004).

Chitosan is produced by the deacetylation of chitin, which is known to be the second most abundant carbohydrate polymer after cellulose (Rinaudo, 2006). Due to its chemical constitutions, chitosan, together with its derivatives like oligochitosan, are cationic, nontoxic, biodegradable and biocompatible compounds, and also possess antifungal effects on various plant pathogenic bacteria and fungi (Zhang et al., 2011; Bautista-Baños et al., 2006, 2013). In previous studies, chitosan was used to preserve fruit quality (Arnon et al., 2014; Han et al., 2014; Liu et al., 2014), and control postharvest decay of horticultural crops caused by fungal pathogen, such as *Botrytis cinerea* (Xu et al., 2007; Lopes et al., 2014), *Colletotrichum gloeosporioides* (Muñoz et al., 2009; Bill et al., 2014), *Alternaria kikuchiana* and *Physalospora piricola* (Meng et al., 2010), *Rhizopus stolonifer*, (Ramos-García et al., 2012), *Monilinia fructicola* (Yang et al., 2012) and *P. expansum* (Wang et al., 2014). Chitosan could act directly on the microorganisms as a fungistatic or fungicidal treatment, but it has also the potential to induce resistance in fruit. It has been well documented that chitosan can elicit the production of reactive oxygen species (Zeng et al., 2010), increase the enzyme activities of catalase, peroxidase, chitinase and β-1,3-glucanase (Meng et al., 2010; Wang and Gao, 2013), and promote the expression of defense-related genes (Ma et al., 2013; Landi

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et al., 2014). However, less is understood about the regulatory genes which are active in response to microbial attack in chitosan treated fruit, especially at a systemic biological level.

Two-dimensional gel electrophoresis (2-DE)-based proteomic analysis provides a systematic approach to understand the cascade of reactions and biological networks subject to complex regulatory mechanisms under diverse postharvest conditions (Hertog et al., 2011; Pedreschi et al., 2013). Therefore, the objectives of the present study were (i) to investigate the effects of chitosan on resistance induction in apple fruit to control blue mold decay caused by *P. expansum*, and (ii) to obtain insights into the changes in protein profiles of apple fruit induced by chitosan treatment and (iii) to infer the underlying molecular mechanisms related to this treatment.

2. Materials and methods

2.1. Fruit

Apples (*Malus. domestica* Borkh, cv. Fuji) were harvested at commercial maturity from an orchard in Fengxian, Jiangsu Province, China and selected for uniformity of size, color and ripeness, and absence of apparent injury or infection. Prior to use, the fruit surfaces were disinfected in 0.1% sodium hypochlorite for 1 min, thoroughly washed with water and air-dried at room temperature.

2.2. Pathogen inoculums

P. expansum provided by China General Microbiological Culture Collection Center was cultured on potato dextrose agar (PDA) at 25 °C for 7 days. Conidia of the fungi were obtained by flooding the cultures with sterile distilled water. The conidial suspension was filtered through three layers of cheesecloth and adjusted to a concentration of 1×10^6 conidia per milliliter with a hemocytometer.

2.3. Effect of chitosan treatment on blue mold decay

Chitosan, with an average deacetylation of 93% and molecular weight of 100 kD (Golden-Shell Biochemical, Yuhuan, China), was dissolved in 2% acetic acid by stirring at room temperature overnight to obtain 1% chitosan/acetic acid solutions. Chitosan treatment was carried out at room temperature by dipping the apples in 1% chitosan/acetic acid solution or 2% acetic acid solution (as control) for 10 min and allowed to air dry at room temperature (Wang et al., 2014). After air drying, apple fruit were put into plastic baskets, overlapped with plastic wrap and stored at 20 °C with a relative humidity of 95% for 15 days. Each treatment contained three replicates with 20 fruits per replicate. Fruit quality and proteomic analysis were conducted at 5, 10 and 15 days after storage.

Effects of chitosan treatment on blue mold decay of apple fruit were evaluated according to the methods described by Cao et al. (2013), with some modifications. Briefly, three uniform wounds (5 mm diameter and approximately 3 mm deep) were made at the equator of chitosan-treated or non-treated apple fruit using the tip of a sterile cork borer. An aliquot of 30 μL of *P. expansum* suspension (1×10^6 spores/mL) was inoculated into each wound. After air drying, apple fruit were put into plastic baskets, overlapped with plastic wrap and stored at 20 °C with a relative humidity of 95% for 15 days. Each treatment contained three replicates with 20 fruits per replicate. Lesion diameters were measured at 5, 10 and 15 days after inoculation and patulin contents were determined at the end of storage.

2.4. Measurement of fruit quality traits

Weight loss was recorded by subtracting final weight from the initial weight of the apple fruit and then expressed as percent weight loss with reference to the initial weight. For total soluble solids (TSS) content and titratable acidity (TA) determinations, apple fruit were homogenized with motar and pestle. After centrifugation at 9000 × *g* for 15 min at 4 °C, the corresponding supernatants were collected. TSS content was estimated by using a WAY-2S Refractometer (Precision & Scientific Instrument, Shanghai, China). TA was measured by titrating with 0.1 M NaOH to pH 8.1, and the results were expressed as malic acid (%). Each treatment was replicated three times.

2.5. Patulin analysis

Decayed portion of apple fruit was collected for patulin measurement. Following the method of Morales et al. (2007), patulin was extracted with ethyl acetate by vigorous mixing. The resulting solvent extracts were combined and then cleaned up by 1.5% sodium carbonate solution. The upper ethyl acetate layer was evaporated to dryness under vacuum. Afterwards, the residues were dissolved in 1 mL of 0.2 mol/L acetic acid solution completely and passed through a 0.22 μm Millipore syringe filter before high-performance liquid chromatography (HPLC) analysis.

Liquid chromatography assay was performed on a Shimadzu SPD-M20A HPLC system (Shimadzu, Kyoto, Japan) equipped with a DAD detector. Separation was conducted at room temperature on a Shim-pack VP-ODS C18 column (250 × 4.6 mm). The mobile phase was water: acetonitrile (90: 10, v/v) at a flow rate of 1 mL min^{-1}. The injection volume was 20 μL, and the detection wavelength was 276 nm. Standard curves of reference compound (Sigma, Shanghai, China) were used to quantify the patulin content.

2.6. Preparation of protein extracts

Proteins were extracted by using the phenol extraction protocol described by Yuan et al. (2014). Approximately 5 g of fruit samples were ground into a fine powder in liquid nitrogen using a mortar and pestle. The resulting powder was suspended in extraction buffer (0.7 M sucrose, 0.1 M KCl, 0.5 M Tris–HCl, 50 mM EDTA, 1% PVPP and 40 mM DTT, pH 8.5). After vortexed for 1 h at 4 °C, the resulting homogenate was mixed with equal volume of 1 M Tris-saturated phenol (pH 7.9, Sunshine Biotechnology, Nanjing, China) and kept at −20 °C for 30 min with vortexing every 10 min. After centrifugation at 10,000 × *g* for 30 min at 4 °C, the upper phenol phase was collected and the aqueous phase was re-extracted once as above. The pooled phenol phases were mixed with 5 volumes of ice-cold 0.1 M ammonium acetate in methanol and incubated at −20 °C overnight. The mixture was centrifuged at 10,000 × *g* for 30 min at 4 °C. The resulting pellets were washed twice with ice-cold methanol containing 0.1 M ammonium acetate followed by twice with ice-cold acetone. The pellet was then vacuum-dried and dissolved in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT and 1% CA pH 3–10. Protein concentration was measured based on Bradford method using BSA as the standard.

2.7. 2-DE and image analysis

Two-dimensional electrophoresis and image analysis were performed as described by Cai et al. (2014). IPG strips (pH 3–10, 17 cm) were passively rehydrated in 300 μL of lysis buffer containing 700 μg of protein for 12 h. Isoelectric focusing was performed in a Protean IEF Cell (Bio-Rad, Hercules, CA). Afterwards, strips were equilibrated for 15 min in 10 mL equilibration buffer (7 M urea, 2% SDS, 0.075 M Tris–HCl, pH 8.8, 30% glycerol) containing 1% DTT, and followed by another 15 min in 10 mL equilibration buffer containing 1.5% iodoacetamide. The second-dimensional SDS-PAGE was performed with 12% polyacrylamide gel in an Ettan DALT system (GE Healthcare, Piscataway, NJ). Protein spots in 2-DE gels