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Combination of chitosan and salicylic acid to control postharvest green mold caused by *Penicillium digitatum* in grapefruit fruit

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ABSTRACT

Green mold caused by *Penicillium digitatum* is critical diseases for grapefruit fruit. In this study, the alone or combined effects of chitosan and salicylic acid on the control of green mold decay in grapefruit fruits were investigated. The results showed that combination of chitosan with SA treatment provided a more effective control of green mold than alone applying chitosan or SA. chitosan plus SA significantly reduced lesion diameter, disease incidence, while the content of endogenous SA much higher than those in treatment alone or control fruit. The combined treatment enhanced the chitinase, β-1,3-glucanase, peroxidase, phenylalanine ammonia-lyase and polyphenoloxidase activities and stimulated the synthesis of total phenolic compounds content. Moreover, the applications of alone or combined did not impair fruit postharvest quality such as firmness, weight loss and TA, while increased ascorbic acid and total soluble solids contents in grapefruit fruits. These result suggest that the use of chitosan combined SA treatment was effective in activating disease resistance against green mold, and providing a longer storage life with acceptable postharvest quality in grapefruit fruit.

1. Introduction

In citrus fruit, various postharvest pathogens caused losses account for nearly 50% of the wastage, which occur at different storage stages after harvest (Ladanyia and Ladaniya, 2010). The most severe postharvest fungal diseases of citrus fruit are green mold caused by *Penicillium digitatum*. Synthetic fungicides, such as prochloraz, imazalil, thiabendazole, and pyrimethanil are used worldwide to control green molds in postharvest citrus fruits (Hao et al., 2011). Although the most effective way to controlling the postharvest disease is synthetic chemical fungicides, because of the occurred fungicide resistance in pathogens and strengthen the awareness of food safety problems caused by fungicides residues, so we need to explore non-toxic and effective ways to control the harvested diseases (Guo et al., 2014). Recently, biologically active natural products have started to be an effective alternative to synthetic fungicides.

Induction of resistance to pathogen is a promising approach for controlling plant diseases, providing long-term systemic resistance to abroad spectrum of pathogen (Walling, 2001). Salicylic acid (SA) is an phytohormone having key roles in different aspects of plant growth and development (Asgharia and Aghdam, 2010). It is also a signaling molecule which induces biosynthesis of defense compounds such as polyphenols or pathogenesis-related proteins (Yang et al., 2011). Many research have reported exogenous SA at nontoxic concentrations enhancing resistance to pathogens and inhibiting decay incidence of many fruits. The recent studies indicated that significant efficacy of biocontrol agents against green mold decay is induced by SA application in citrus fruits (Moscoso-Ramírez and Palou, 2013; Zhou et al., 2014).

Chitosan, a deacetylated derivate of chitin, is a high-molecular-weight cationic linear polysaccharide composed of D-glucosamine and, to a lesser extent, N-acetyl-D-glucosamine with a b-1,4-linkage (Shahidi, 2007). Due to its chemical constitutions, chitosan, together with its derivatives like oligochitosan, are cationic, nontoxic, biodegradable and biocompatible compounds; and have been studied for efficacy in inhibiting decay and extending shelf life of fruits (Wang and Gao, 2013). Chitosan and its derivatives have been shown to inhibit the growth of a wide range of fungi (Deng et al., 2015). They can also trigger defensive mechanisms in plants and fruits against infections caused by several pathogens (Zhou et al., 2016).

In previous studies, chitosan was used to preserve fruit quality (Wang and Gao, 2013), and control postharvest decay of horticultural fruit caused by green mold (Shao et al., 2015; Waewthongrak et al., 2015). In addition of the direct antifungal effect, many researches focused on the potential of inducing defense response in fruit. It has been

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well documented that they can elicit the production of reactive oxygen species (Zeng et al., 2010), increase the enzyme activities of phenylalanine ammonialyase, polyphenoloxidase, peroxidase, chitinase and β-1,3-glucanase (Meng et al., 2010; Shen and Yang, 2017), and promote the expression of defense-related genes (Meng et al., 2012). However, few reports have exhibited the effects of a combination of chitosan and SA on inducing disease resistance and postharvest food quality in citrus fruits.

The objectives of the present work were to investigate (a) the effects of postharvest chitosan and SA used alone or combined, on controlling postharvest green mold caused by *Penicillium digitatum* in grapefruit fruits; (b) the effects of postharvest applications containing chitosan, SA and the combination of both on the key of host defense enzymes; (c) the effects of chitosan and SA, alone or combined, on postharvest quality of grapefruit fruit during storage.

2. Materials and methods

2.1. Fruit

Grapefruit fruit (*Citrus paradise* Macf.) were harvested at firm-mature stage from plants grown in a commercial field at Kunming, China, then packaged in cartons and transported to the laboratory within 24 h, and subsequently stored at room temperature (20 °C, 85–95% relative humidity).

2.2. Pathogen inoculum

The pathogen *Penicillium digitatum* was originally isolated from an infected grapefruit fruit (*Citrus paradise* Macf.) and maintained on potato dextrose agar (PDA, containing the extract from 200 g boiled potato, 20 g glucose and 20 g agar in 1 L of distilled water) at 28 °C. Spore suspension of the pathogen was prepared by activated fungal spores of *P. digitatum* with sterile distilled water. Spore concentration was determined by hemocytometer measurement and adjusted as required with sterile distilled water.

2.3. Treatments

Fruit were selected for similar size, ripeness and without external damage, and then divided at random in four groups. Grapefruit fruit were immersed for 2 min in 2% (v/v) sodium hypochlorite for surface-disinfected, then, washed with running tap water, and air-dried at room temperature. The concentration of chitosan and salicylic acid was according to Shen and Yang (2017). Treatments were subject as mentioned below: (1) Control: Fruit were dipped with sterile-distilled water for 10 min; (2) Chitosan treatemt: Fruit were dipped with 10 g L^{-1} (w/v) chitosan for 10 min; (3) SA treatment: Fruit were dipped with 2 mM SA for 10 min; (4) SA + Chitosan treatment: Fruit were dipped with 2 mM SA for 5 min, after air-drying for 1 h, fruit were again dipped with 10 g L^{-1} (w/v) chitosan for 5 min. All treated fruits were air-dried, kept in cartons and stored at room temperature for subsequent experiments. There were triplicate samples consisting of 20 fruits per replicate and all assessment were conducted twice. Chitosan and salicylic acid were purchased from Sigma-Aldrich (USA).

2.4. Effects of chitosan and SA on disease incidence and lesion diameter of *P. digitatum* in grapefruit fruit

For the efficacy of chitosan and SA dipped treatment on disease controlling *P. digitatum*, fruits was wounded with a sterilized borer (3 mm deep × 2 mm wide) at three points around the equator of each fruit. Then 20 μL of 1×10^4 spores ml^{-1} suspension of *P. digitatum* was inoculated into each wound. After air drying, all fruits were separately packed in plastic bags and incubated at room temperature for 6 days. The disease incidence and lesion diameter were recorded 2 day intervals after inoculation. There were three replicates per treatment with 10 fruit.

2.5. Sample collecting

Three grams of tissues were detached from 4 to 8 mm below the skin around the equator of the fruit at 7, 14, 21, 28 and 35 d after treatment. Each sample was packed in aluminum foil individually and frozen in liquid nitrogen immediately, and kept at −80 °C until biochemical analysis.

2.6. Effects of chitosan and SA on endogenous SA in grapefruit fruit

Endogenous SA was extracted using the method of Zhang et al. (2003). Detection of SA was done according to Zhang et al. (2015) with suitable modifications. Quantitative detection of endogenous SA was made using a Shimadzu SPD-M20A HPLC with Agilent TC-C18 column (4.6 × 250 mm). The supernatant was filtered through a 0.45-um Millipore filter. Samples were eluted with methanol and acetic acid (1:1) at a flow rate of 1 mL min^{-1} and detected using a Shimadzu Fluorescence Detector with excitation and emission wavelengths of 310 and 415 nm, respectively. Ten microliters of each sample were injected into the column. Identification of SA was achieved by comparing its retention time with that from authentic standard SA. The quantity of SA was computed from the standard curve made with known concentrations of SA and expressed as μg g^{-1} FW. Three independent replicates were conducted for each treatment.

2.7. Effects of chitosan and SA on total phenolic compound content and defense-related enzyme activities in grapefruit fruit

Total phenolics compounds content was determined spectrophotometrically using Folin-Ciocalteu reagent following the method of Yang et al. (2011). The total phenolics content of sample was expressed as grams of gallic acid equivalent (GAE) per g of fresh weight (FW).

Peroxidase (POD) and polyphenoloxidase (PPO) activities were measured according to the method of Zhou et al. (2014). A fresh fruit peel was mixed with 5 mL of ice-cold sodium phosphate buffer (100 mM, pH 7.8). The results were expressed as units (U) per gram fresh weight (FW). One unit of POD activity was defined as the amount of enzyme which caused a change of 1 in absorbance per minute at 470 nm. One unit of PPO activity was defined as the amount of enzyme which caused a change of 1 in absorbance per minute at 420 nm.

Phenylalanine ammonia-lyase (PAL) activity was analyzed using the method of Zhou et al. (2014). The result was expressed as units (U) per gram of fresh weight (FW). One unit of PAL activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 at 290 nm per hour.

Chitinase (CHI) and β-1,3-glucanase were measured according to the methods described by Luo et al. (2012). The result was expressed as units (U) per gram of fresh weight (FW). One unit of CHI activity was defined as the amount of enzyme required to catalyze the production of 1×10^{-9} μg of N-acetyl-D-glucosamine per second. For β-1,3-glucanase, one unit of enzyme activity was defined as the amount of enzyme that produced a reducing sugar equivalent to 1×10^{-9} mol glucose equivalent per second.

2.8. Effects of chitosan and SA on postharvest quality parameters in grapefruit fruit

During storage, fruit samples were collected from five fruits randomly chosen from each group. The firmness was measured using a fruit pressure tester (GY-2, Huier Industry Co. Ltd., Hangzhou, China) fitted with a 3.5 mm diameter probe. Weight loss was determined per treatment and was recorded initially and each time during storage. Weight loss was calculated as: weigh loss = $(Wi - Wf)/Wi \times 100\%$, *Wi*