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Control of brown rot on nectarines by tea polyphenol combined with tea saponin

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ABSTRACT

Postharvest peach fruit are susceptible to fungal decay. To search for effective alternatives to currently used fungicides for disease control, the *in vitro* and *in vivo* antifungal activities of tea polyphenol (TP) and tea saponin (TS) individually or their combination were evaluated against *Monilinia fructicola* isolated from infected nectarine fruit in this study. The *in vitro* experiments showed that both TP and TS inhibited the mycelial growth in a dose-dependent manner and their combination exhibited an additive or synergistic antifungal interaction with the cotoxicity factor (CTF) exceeding −20. The *in vivo* experiments showed that they both effectively controlled brown rot on inoculated nectarine fruit and their combination (TP: TS, 1:2) significantly improved the controlling effect. Our results also revealed that TP and TS induced the activities of phenylalanine ammonia-lyase (PAL), peroxidase (POD), polyphenoloxidase (PPO), chitinase and β-1,3-glucanase in inoculated nectarine fruit and their combination strengthened the induction. These findings suggested that the combination of TP and TS could effectively control brown rot on postharvest fruit and that the mechanism of the control might be attributed to the induction of defensive enzyme activities.

1. Introduction

Brown rot is the most important postharvest disease of stone fruit. *Monilinia fructicola* (G. Wint.) Honey is one of the most important causal agents of brown rot of peach fruit (Ogawa and English, 1995; Emery et al., 2002; Zhu et al., 2005). Brown rot frequently causes commercially significant losses. Both industry and consumers urgently demand effective strategies for prolonging shelf life of peach fruit. The disease control is still mainly dependent on the use of chemical synthetic fungicides, such as carbendazim, benomyl, imidacloprid and thiophanate-methyl (Korsten, 2006). However, the application of these fungicides has been increasingly curtailed by the development of pathogen resistance to many key fungicides, the lack of replacement fungicides, negative public perception regarding the safety of pesticides and consequent restrictions on chemical fungicide use. Therefore, it is urgent to find new, safe and effective fungicides to control postharvest diseases of peach fruit.

The control of postharvest fruit decay with natural extracts has been increasingly noticed as a novel trend in biological preservation in recent years. Several studies have shown that the extracts of lemon (Viuda-Martos et al., 2008), eugenol (Amiri et al., 2008), *Putranjiva roxburghii* (Tripathi and Kumar, 2007) and *Cinnamomum osmophloeum* leaf (Bajpai et al., 2009) have antifungal activity against pathogenic fungi and postharvest fruit decay has been effectively controlled by their extracts.

Green tea, comes from the leaves of *Camellia sinensis* L., is the most widely consumed beverage in China for its health benefits, and it is rich in polyphenolic compounds known as tea polyphenol (TP). TP has many bioactivities, such as anticarcinogenic properties (Jankun et al., 1997), preventing cardiovascular diseases (Cheng, 2006) and anti-oxidation (Almajano et al., 2008). Recently, TP has attracted much attention for antimicrobial activity. Many studies have reported that TP has displayed antimicrobial activity against human and animal disease-related bacteria, plant pathogenic bacteria (Fukai et al., 1991), food-borne bacteria (Bandyopadhyay et al., 2005), pathogenic viruses (Xu et al., 2008) and pathogenic fungi (Hirasawa and Takada, 2004), which demonstrate its potential as a preservative in food industry, especially in the field of fruit preservation. However, there has been little research on the control of fungal pathogens, such as *M. fructicola*, in postharvest fruit.

Tea saponin (TS) is extracted from seed of plant species in genus, *Camellia* of *Theaceae*. It can enhance efficiency, solubilization and attenuated poison of pesticide as a wettable agent of wettable powder pesticide. Therefore, TS has been widely used in the area of pesticides as the main component of environment-friendly pesticide additives. The antimicrobial activity of TS has recently been noticed. Several studies have reported that TS does not only exhibit

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good antimicrobial activity against bacteria, fungi, yeast and viruses (Li et al., 2009; Yang and Zhang, 2012), but also enhances the antimicrobial activity of fungicides (Hao et al., 2010; Yang and Zhang, 2012) and reduces the necessary amounts of these fungicides.

The objectives of the present study are to evaluate the following: (1) the *in vitro* antifungal activities of both TP and TS against *M. fructicola* and their synergistic interaction; (2) the *in vivo* antifungal activities of TP and TS individually or their combination on inoculated fruit; and (3) the mechanism underlying their antifungal properties.

2. Material and methods

2.1. Chemicals and fruit

Green tea polyphenol (TP, purity of 98%) was purchased from Nanjing Qingze Medical Technological Development Co. Ltd (China). Tea saponin (purity of 98%) was purchased from Hunan Geneham Biomedical Technology Ltd. All other chemicals and reagents were of analytical grade.

Peach (*Prunus persica var. nectarina cv.* Shuguang) fruit used in this study were harvested at commercially maturity from Wuhan, China. Fruit were free of damage and fungal infection. They were selected by hand for uniformity of size and ripeness, without chemical postharvest treatment.

2.2. Fungal pathogen

M. fructicola was isolated from infected nectarine fruit with typical brown rot symptoms, purified and identified. *M. fructicola* conidia were collected and suspended in sterile water and filtered through a gradient of cheesecloth. The concentration of conidial suspension was determined using a hematocytometer. The conidial suspension was distributed onto water agar amended with 100 μg mL^{-1} streptomycin sulfate in Petri dishes. A hyphal tip from a germinating conidium was transferred aseptically to potato dextrose agar (PDA) and single spore cultures were used for further experimentation.

2.3. In vitro antifungal assays

Antifungal assays of TP and TS were performed by mycelial growth assay as described previously (Yen et al., 2008) with slight modifications. Serial concentrations of TP and TS (0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 mg mL^{-1}) were tested for action against mycelial growth of *M. fructicola*, using sterile distilled water as a control. TP and TS solutions were respectively added to sterilized PDA at 40–50 °C and poured into Petri dishes (9 cm in diameter). A mycelial dish of approximately 10 mm in diameter was cut from the edge of 3-day-old culture of *M. fructicola* and was placed at the center of each Petri dish. The inoculated plates were incubated at 25 °C for three days until the growth in the control treatment plates reached the edge of the Petri plates. Each test was replicated four times and each treatment within a replication was repeated five times. The antifungal index was calculated as follows: Antifungal index (%) $= (1 - D_a/D_b) \times 100$, where D_a is the diameter of the growth zone on treated PDA (cm), and D_b is the diameter of the growth zone on untreated PDA (cm). The EC_{50} value (the concentration of each chemical that inhibited 50% of mycelial growth) was calculated by probit analysis with the help of probit package of SPSS 11.5 software.

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined as previously described (Kubo and Himejima, 1992) with slight modifications, respectively. The tested compounds were added to the sterilized potato dextrose (PD) media to prepare serial 2-fold dilutions in a concentration range from 2.5 to 640 mg mL^{-1}. Then, 180 μL of the serial dilutions and 45 μL of fungal liquid cultured media were added to 96-wells microplates, using sterile PD media as control. After inoculating, the microplates were incubated at 28 °C. MIC is the lowest concentration of each tested compound that resulted in no visible mycelial growth after 48 h of incubation. MFC was the lowest concentration of each tested compound in which no recovery of a microorganism was observed after seven days.

2.4. Evaluation of in vitro synergistic interaction

The evaluation of the synergistic interaction of TP and TS was conducted according to Mansour et al. (1966). The EC_{50} solutions of TP and TS were respectively mixed at the ratios (volume: volume) of 1:5, 1:2, 1:1, 2:1 and 5:1. The combined effect of TP and TS was expressed as the cotoxicity factor (CTF) which was calculated according to the following equation given by Mansour et al. (1966): $CTF = (O_o - O_e)/O_e \times 100$, where O_o is the actually observed antifungal index of the combination by mycelial growth assay and O_e, the expected antifungal index of the combination, is the sum of antifungal index produced by each agent in the combination. O_e is calculated according to the equation: O_e = the antifungal index of $A \times P_A$ + the antifungal index of $B \times P_B$, where A and B represent the two components, and P_A and P_B represent the ratio of the components in the combination. CTF was used to differentiate the result into three categories: a positive factor of 20 or more was considered as synergism, while a negative factor of 20 or more considered antagonism, and any intermediate value (i.e. between −20 and +20) considered additive. Each test was replicated four times and each treatment within a replication was repeated five times.

2.5. Inoculation and treatment procedures

Nectarine fruit used in this study were washed with running tap water. Their surfaces were sanitized with 2% sodium hypochlorite for 2 min, rinsed twice in sterile distilled water and dried in ambient air. The fruit were made a single puncture on the equator with a stainless steel rod with a 4-mm-wide and 5-mm-long tip (Eckert and Brown, 1986). Each puncture was inoculated with 25 μL of inoculant containing 5×10^4 conidia mL^{-1} of *M. fructicola* and held at 25 °C for 2 h. Then, the fruit were again inoculated with 50 μL of the following treatment solutions at the same puncture. The treatment solutions were as follows: carbendazim (5.0 mg mL^{-1}), TP (8.5 mg mL^{-1}), TS (8.5 mg mL^{-1}), the combination of TP and TS (R_1, 8.5 mg mL^{-1}), the combination of TP and TS (R_2, 17.0 mg mL^{-1}) and the combination of TP and TS (R_3, 25.5 mg mL^{-1}). Control fruit (CK) were treated with sterile distilled water. The fruit were stored at 25 °C with 85% RH for four days. Disease incidence was assessed by counting infected fruit and disease severity was assessed by measuring diameter of rotten area using a caliper. There were 20 peaches in each treatment and each treatment was replicated four times. The experiment was repeated three times.

2.6. Enzyme analysis

Nectarine fruit were washed, sanitized and punctured according to the method described above. Each puncture was respectively inoculated with 25 μL of inoculant containing 5×10^4 conidia mL^{-1} of *M. fructicola* and 50 μL of the treatment solutions. The treatments were as follows: CK group (sterile distilled water + sterile distilled water), *M. fructicola* group (*M. fructicola* + sterile distilled water), TP group (*M. fructicola* + 8.5 mg mL^{-1} of TP), TS group (*M. fructicola* + 8.5 mg mL^{-1} of TS) and TP + TS group