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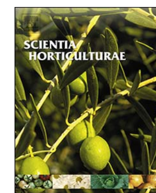

Research paper

Effects of quercetin on postharvest blue mold control in kiwifruit

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A B S T R A C T

The effects of quercetin on inhibiting *Penicillium expansum* infections of 'Qinmei' kiwifruit during postharvest storage were evaluated. Severe damage to *P. expansum*'s mycelial structure was observed by transmission electron microscope at a quercetin concentration of 0.25 mg/mL. With the quercetin treatment, kiwifruit infected with *P. expansum* had higher chitinase, β-1,3-glucanase, phenylalanine ammonialyase, polyphenol oxidase and peroxidase activities, as well as *nonexpressor of pathogenesis-related gene 1*, *pathogenesis-related gene 1*, *chitinase* and *β-1,3-glucanase* expression levels, compared with those of kiwifruit only treated with quercetin or inoculated with *P. expansum*. Thus, the inhibitory effects of quercetin on the *P. expansum* blue mold may be associated with its toxic properties against fungal pathogens and inhibition of defense responses.

1. Introduction

Kiwifruits have been broadly cultivated in many countries and are becoming an important fruit worldwide. Kiwifruits are classified into two species, *Actinidia deliciosa* and *Actinidia chinensis* (Ferguson, 1991; Ferguson et al., 2008), based on their capabilities. They are recognized as climacteric fruit because of the autocatalysis of ethylene production. This production promotes enzymatic activities, resulting in the softening and ripening of kiwifruit. *Penicillium expansum* or blue mold infection is a main cause of kiwifruit spoilage after harvest (Duan et al., 2013; Hu et al., 2011; Neri et al., 2010). *P. expansum* infection occurs during storage on wounds produced during harvesting and handling, as well as sites having other primary fruit pathogens. Over-mature or long-stored kiwifruit are more susceptible to *P. expansum* infections (Hur et al., 2005). Synthetic fungicides are currently used to control kiwifruit infections (Bardas et al., 2010). However, public concerns regarding the potential impacts of fungicidal residues on human health and the environment are encouraging the development of safe and environmentally friendly methods for preventing *P. expansum* infections (Bi et al., 2007; Ruiz-garcía and Gómez-plaza, 2013; Terry and Joyce, 2004). Recently, treatments with hot water, harpin and oxalic acid have been used to stimulate defense responses in kiwifruit (Chen et al., 2015; Deng et al., 2015; Tang et al., 2015). However, compared with other fruits, such as citrus and apple, the induction of disease resistance in kiwifruit during the postharvest period has received little attention or documentation (Palou et al., 2008; Quaglia et al., 2011).

Quercetin, an important antioxidant flavonoid, is generally present in vegetables, fruits, teas and wines, as well as in onion (Erlund, 2004). Quercetin possesses beneficial health functions, especially the capability of scavenging highly reactive species, such as peroxynitrite and hydroxyl radicals (Boots et al., 2008; Harwood et al., 2007; Russo et al., 2012). Additionally, quercetin and its derivatives have effective antifungal activities against the dermatophyte *Trichophyton rubrum* and *Candida albicans* cultures (Bitencourt et al., 2014; Tempesti et al., 2012). Grape pomace extract, containing quercetin, also has an antifungal activity against *Botrytis cinerea* (Mendoza et al., 2013). In addition, exogenous applications of quercetin on fruit induce resistance to blue mold during apple production (Sanzani et al., 2009a, 2009b, 2010). The objectives of this research were to evaluate the antifungal activities of quercetin against *P. expansum in vitro*, as well as its effects on, and the mechanisms involved in, inducing resistance in kiwifruit. The results will be helpful in extending the shelf-life of kiwifruit, and in the development of a natural and safe antifungal agent for vegetable and fruit preservation.

2. Materials and methods

2.1. Experiment materials

Kiwifruits (*Actinidia deliciosa* cv. Qinmei) were purchased from a local farmer's orchard in Zhouzhi (Shaanxi Province, China) and immersed for 2 min in 2% (v/v) sodium hypochlorite solution for surface disinfection. Then, they were washed with running tap water and air-dried at 23 ± 2 °C.

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Dimethyl sulfoxide (DMSO) was used to dissolve quercetin, which was then further diluted with sterile distilled water. The final DMSO concentration in solution was 0.8% (v/v).

The 'Quercetin' was purchased from Dalian Meilune Biotechnology Co., Ltd, and purity ≥ 98%.

2.2. Pathogen

An isolate of *P. expansum* obtained from rotted kiwifruit was incubated on potato dextrose agar (PDA) medium at 25 °C for 7 days. Sterile distilled water containing 0.05% Tween-20 was used to wet the surface of the PDA culture before the conidia were scraped using a sterile loop. The spore concentration was adjusted to 1×10^5 conidia/mL using a hemocytometer.

2.3. Mycelial growth of P. expansum

The growth method was based on the previous study of Sanzani et al. (2009a, 2009b) with some modifications. Aliquots of 20 µL *P. expansum* spore suspension were centrally inoculated in holes (5 × 5 mm) on individual petri dishes (90 mm in diameter) containing PDA supplemented with different concentrations of quercetin (0, 0.25, 0.5 or 1.0 mg/mL). Each plate was turned over and incubated at 25 °C in the dark. Colony diameters (mm) were recorded 14 days post-inoculation. There were five replicates for each concentration, and the experiment was repeated twice.

2.4. Observations of the hyphal ultrastructures

The preparation of hyphae was performed as in Zhang et al. (2011). Hyphae (2 g wet weight) were transferred to an Erlenmeyer flask (50 mL) containing 30 mL sterile distilled water with 0.25 mg/mL quercetin and to sterile distilled water containing 0.8% DMSO, which served as the control. The mycelial fragments were sampled after 12 h of incubation at room temperature (23 ± 2 °C) and washed three times with sterile double-distilled water.

The ultrastructure was analyzed using the modified method of Shao et al. (2013). Mycelial fragments were placed in 2.5% glutaraldehyde in 0.1 mol/L potassium phosphate buffer (pH 7.2) for 24 h at 4 °C. They were washed five times, 15 min each, using the same buffer. Then, the samples were fixed in 1% osmium tetroxide for 2 h, washed five times as before, and then dehydrated for 30 min in a graded series (30, 50, 70, 80, 90 and 100%) of ethanol before being embedded in a LR White resin. Ultrathin sections were cut using a Leica EM UC7 ultra-microtome (Leica, Mikrosysteme, Germany) and then dyed by lead citrate for 10 min and uranyl acetate for 8 min. An HT-7700 transmission electron microscope (Hitachi, Japan) was used to examine the ultrastructures of each dyed specimen.

2.5. Postharvest treatment and inoculation

Based on our preliminary study, 0.10, 0.25 and 0.50 mg/mL quercetin were chosen as the desired concentrations to evaluate the inhibitory effects on the blue mold of kiwifruit. Four wounds (4 mm deep × 5 mm wide) were made on the equatorial side of each fruit with a sterile spike. Each well was injected with 25 µL of a quercetin concentration (0.10, 0.25 or 0.50 mg/mL). Injected sterile distilled water containing 0.8% DMSO served as a control. The fruit were placed at 23 ± 2 °C for 24 h, and then 20 µL of spore suspension was injected into each wound. The treated fruits were stored in polyethylene-lined plastic bags at 23 ± 2 °C. Lesion diameters and disease incidence levels on each kiwifruit were recorded 7 days after inoculation. Lesion diameters were measured by a crossing method using only those wounds that were infected. Disease incidence was recorded as the percentage of fruit with obvious disease symptoms. Each treatment included three replicates, which consisted of 30 fruits.

Kiwifruit were wounded as described above and randomly divided into four groups of 50 fruits each: (1) Control, fruits inoculated only with 25 µL of 0.8% DMSO; (2) *P. expansum*, fruits inoculated only with 20 µL of *P. expansum*; (3) Quercetin, fruits only treated with 25 µL of quercetin at 0.25 mg/mL; and (4) Quercetin + *P. expansum*, fruits infected with 20 µL of *P. expansum* 24 h after treatment with 25 µL of 0.25 mg/mL quercetin. After treatment, all of the fruits were stored in polyethylene-lined plastic bags at 23 ± 2 °C. Tissue samples from five fruits in each replicate were collected at 0, 12, 24, 36, 48, 72 and 96 h after infection. The samples were mixed and immediately frozen in liquid nitrogen, then ground in a chilled grinding apparatus (IKA A11) to a powder and stored at −80 °C before the enzyme activity and gene expression analyses. Each treatment was replicated three times, and the whole experiment was conducted twice.

2.6. Assay of defensive enzyme activities

To determine the defensive enzyme activity levels, the enzymes were extracted using 1 g of the powder dissolved in 5 mL of extraction buffer as follows: 0.05 mol/L sodium phosphate buffer [pH 7.8, contained 1 mmol Ethylene Diamine Tetraacetic Acid (EDTA), 2% Polyvinyl-polypyrrolidone (PVPP) and 0.3% TritonX-100) for polyphenol oxidase (PPO), 0.1 mol/L sodium acetate buffer (pH 5.5, contained 1 mmol PEG6000, 4% PVPP and 1% TritonX-100) for peroxidase (POD), 0.1 mol/L sodium borate buffer (pH 8.8, contained 4% PVPP, 2 mmol EDTA and 5 mmol β-mercaptoethanol) for phenylalanine ammonialyase (PAL), 0.1 mol/L sodium acetate buffer (pH 5.0, contained 1 mmol EDTA, 2% PVPP, 5 mmolβ-mercaptoethanol) for chitinase (CHI) and β-1,3-glucanase (GLU). Static extraction 1 h after the flesh sample and the extract were fully mixed, then centrifuged at 12,000 × *g* for 20 min at 4 °C. The supernatant fraction served as the crude enzyme source to assay enzymatic activities. Each sample was analyzed in three replicates.

PPO activity was determined based on the method of Coseteng and Lee (1987). The reaction mixtures were 2.9 mL of 0.05 mol/L citric-phosphoric acid buffer (pH 5.5, containing 0.01 mol/L pyrocatechol), and 100 µL of the crude enzyme solution. The continuous change of the absorbance at A420 nm was determined at once within 3 min, which caused an increase of 1 at A420 per min as one unit of PPO activity and the results were expressed as U/g fresh weight.

POD activity was assayed by the method described by Yu et al. (2014). The reaction mixture consisted of 100 µL of the crude enzyme and 3 mL of 0.3% (v/v) guaiacol. The increase in the absorbance at 470 nm was measured after 200 µL of 0.5 mol/L H_2O_2 was added. The continuous change of the absorbance at A470 nm was determined at once within 3 min, which caused an increase of 1 at A470 per min as one unit of POD activity and the results were expressed as U/g fresh weight

PAL activity was determined according to Zong et al. (2010) with minor modifications. In total, 500 µL of the enzyme extract solution was mixed with 3 mL of borate buffer and incubated with 500 µL of L-phenylalanine (0.05 mol/L) at 37 °C for 1 h. An incubation with 500 µL distilled water instead of L-phenylalanine was used as a reference blank. The reaction was stopped by adding 100 µL of HCl (6 mol/L). One unit of PAL activity was defined as the amount of enzyme that caused an increase of 1 at A290 per hour and expressed as U/g fresh weight.

CHI activity was measured based on the study of Zheng et al. (2011). Here, 500 µL sodium acetate buffer (pH 5.0) was mixed with 500 µL of colloidal chitin (diluted in 95% HCl and 50% ethanol), and then incubated with 500 µL of the crude enzyme extract at 40 °C for 1 h. The crude enzyme solution was boiled for 5 min as a control. Then, 100 µL of 30 g/L desalted snailase was added and incubated at 37 °C for 1 h. Next, 300 µL of 0.6 mol/L potassium tetraborate was added immediately and followed by boiling for 5 min to terminate the reaction. Finally, 2 mL of 100 g/L 4-(dimethylamino) benzaldehyde reagent

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