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The modes of action of epsilon-polylysine (ε-PL) against *Botrytis cinerea* in jujube fruit

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ABSTRACT

Epsilon-polylysine (ε-PL) is a natural antimicrobial and has been widely used as a food preservative. Previous studies showed ε-PL being effective for control of foodborne bacteria; however, little is known about its control effects on postharvest pathogenic fungi and the possible modes of action. Here, we found that ε-PL could significantly inhibit mycelial growth, spore germination and germ tube elongation of *Botrytis cinerea in vitro*, as well as effectively control gray mold in harvested jujube fruit. Further investigation indicates that the modes of action of ε-PL against *B. cinerea* include (i) stimulating the accumulation of intracellular reactive oxygen species (ROS) and reducing the expression of pathogenesis-related genes of the pathogen, (ii) resulting in the leakage of soluble carbohydrates and nucleic acids, as well as the damage of plasma membrane integrity of the fungal cells, (iii) inducing the expression of respiratory burst oxidase homolog (RBOH) genes in ε-PL-treated jujube fruit.

1. Introduction

Botrytis cinerea is a necrotrophic pathogen that causes postharvest decay in various horticultural crops (Williamson et al., 2007). The economic losses caused by this pathogen usually surmount $ 10 billion worldwide each year (Weiberg et al., 2013). In the past decade, chemical fungicides remained the main way to control the postharvest decay in fruits and vegetables (Leroux et al., 2002), and the annual global expenses at *Botrytis* control easily surmounted € 1 billion (Dean et al., 2012). Excessive use of fungicides results in drug resistance of *B. cinerea* (Leroux et al., 2002), and chemical fungicides are harmful to human health as well as the environment. Moreover, an increasing number of regulatory restrictions are put on the use of fungicides (Droby et al., 2009). Therefore, it is important to find some safe and effective antimicrobials to control this tough pathogen instead of chemical fungicides. A number of promising approaches including postharvest application of cinnamic acid (Zhang et al., 2015b), antagonistic yeasts (Qin et al., 2004; Chan and Tian, 2005), and antagonistic yeasts combined with silicon (Qin and Tian, 2005), boron (Shi et al., 2011; Cao et al., 2012a) or heated water (Karabulut et al., 2004; Zong et al., 2010) have been successfully used to control postharvest diseases in various fruits.

Epsilon-polylysine (ε-PL) is a promising natural antimicrobial typically composed of 25 to 35 identical L-lysine residues (Yoshida and Nagasawa, 2003). It was initially isolated from culture filtrates of *Streptomyces albulus* no. 346 and now produced through aerobic fermentation using a mutant derived from *S. albulus* (Bo et al., 2014). ε-PL is stable at high temperatures (Yoshida and Nagasawa, 2003) and has been confirmed to be safe by experiments using rats (Neda et al., 1999; Hyldgaard et al., 2014). Moreover, ε-PL can be degraded to lysine, which is one of the essential amino acid for human (Eagle, 1959), and has an anti-obesity effect since it suppresses fat absorption by inhibiting pancreatic lipase activity (Kido et al., 2003). Based on its antimicrobial activity, stable characteristic and safety, ε-PL has been extensively used for preserving food (Ye et al., 2013; Zhang et al., 2015a), and approved as a natural food preservative by the United States, Japan, Korea and China. However, the effects of ε-PL on fungal pathogens in harvested fruits and its modes of action, is largely unknown. The objective of this study was to investigate the inhibitory effects of ε-PL, an innovative antibacterial preservative, on *B. cinerea* both *in vitro* and in jujube fruit, as well as to further ascertain its modes of action against the pathogen.

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2. Materials and methods

2.1. Fruit

Jujube (*Zizyphus jujuba* cv. Dongzao) fruit at commercial maturity were harvested from an orchard in Beijing and instantly transported to the Institute of Botany, Chinese Academy of Sciences. Fruit with uniform size and without physical injuries or infections were sorted. Prior to use, jujube fruit were disinfected with 2% (v/v) sodium hypochlorite for 2 min, rinsed with tap water, and air-dried.

2.2. Pathogen

B. cinerea (B05.10) was offered by Prof. Paul Tudzynski (Westfaelische Wilhelms-Universitaet Muenster, Germany) and maintained on potato dextrose agar (PDA) plates. Fungal spores were obtained by flooding the plates with sterile distilled water after culturing for 10 d at 22 °C. Spore suspensions were then filtered by four layers of sterile cheesecloth to remove any adhering mycelia. The concentration of the spore suspension was assayed using a hemacytometer.

2.3. Effect of ε-PL against B. cinerea in jujube fruit

Jujube fruit were wounded (3 mm deep × 3 mm wide) using a sterile nail at the equator of each fruit. Then 5 μL of spore suspension at the concentration of 2×10^4 mL^{-1} were inoculated into each wound. The fruit were air-dried for 1 h, and then 20 μL of ε-PL at 1000, 2000, or 4000 mg L^{-1} was added into each wound. Sterile distilled water was used as control. After air-drying for 1 h at room temperature, the treated fruit were placed into plastic boxes with sterile water to maintain a humidity of 95%, and stored at 22 °C. Disease incidence and lesion diameter were measured daily after treatment. Each treatment contained three replicates with 15 jujube fruit per replicate. The entire experiment was repeated twice.

2.4. Effect of ε-PL on mycelial growth of B. cinerea

The effect of ε-PL on mycelial growth of *B. cinerea* was determined on PDA plates. The ε-PL powder was dissolved in sterile water and mixed with PDA to obtain final concentrations of 0, 150, 300, and 450 mg L^{-1}. Spores of *B. cinerea* were diluted to a concentration of 1×10^6 mL^{-1}, and 5 μL spore suspension was added into the center of the PDA plates. The plates were incubated at 22 °C, and the diameters of the colonies were assayed. Each treatment included three replicates, and the entire experiment was repeated twice.

2.5. Assay of spore germination and germ tube elongation

The inhibitory effects of ε-PL on spore germination and germ tube elongation of *B. cinerea* were examined as described previously (Qin et al., 2010). In brief, potato dextrose broth (PDB) media were supplemented with different concentrations of ε-PL at 0, 50, 100, and 150 mg L^{-1}. Spore suspension of *B. cinerea* was added into the PDB medium containing ε-PL to obtain a final concentration of 1×10^6 mL^{-1}. The inoculated PDB media were cultured at 25 °C on a rotary shaker. Spore germination and germ tube elongation were assayed at 2, 4, 6, and 8 h after inoculation. A spore was considered germinated when the length of germ tube was equal to or greater than the diameter of the spore. Germination rate was expressed as the percentage of germinated spores out of the total spores evaluated. Germ tube length was assayed with an ocular micrometer. Approximately 200 spores were randomly observed and the experiment was repeated three times.

2.6. Determination of intracellular ROS and plasma membrane integrity

Intracellular ROS and plasma membrane integrity were evaluated by observing the spores of *B. cinerea* stained with 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich) and propidium iodide (PI; Sigma-Aldrich), respectively. DCFH-DA is an oxidant-sensing fluorescent probe used for detection of intracellular ROS formation, and PI is a dye commonly used to identify membrane integrity of cells (Fish et al., 2000). Spores of *B. cinerea* were cultured in PDB media with different concentrations of ε-PL (0, 150, and 300 mg L^{-1}) for 2, 4, 6, and 8 h at 25 °C. Spores were collected and stained with DCFH-DA or PI for 10 min at 30 °C. Then, the spores were washed twice with phosphate-buffered saline (PBS), and observed under a Leica DM 2500 microscope (Leica, Germany). Three fields of view were randomly chosen for each treatment, and the experiment was repeated twice.

2.7. Detection of cellular leakage

The leakage of cytoplasmic contents from mycelia of *B. cinerea* treated with different concentrations of ε-PL was detected as described previously (Lewis and Papavizas, 1987; Cai et al., 2015) with some modifications. Spores of *B. cinerea* were cultured in PDB media on a rotary shaker for 3 d at 25 °C. After being washed twice with sterile distilled water, the mycelia were resuspended in sterile distilled water containing ε-PL at 0, 150, and 300 mg L^{-1}, and shaken at 25 °C for 1, 2, 3, and 4 h. The mycelia were then filtered and the aqueous solutions were used for determination of leakage of soluble carbohydrates and nucleic acids. The anthrone reagent with glucose as the standard (Morris, 1948) was performed to quantify the release of soluble carbohydrates. Leakage of nucleic acids was measured through detecting the optical density at 260 nm (OD_{260}). Each treatment contained three replicates, and the experiment was repeated twice.

2.8. Total RNA extraction and RT-qPCR

Total RNA of *B. cinerea* was isolated from the mycelia treated with different concentrations of ε-PL using TRNzol Reagent (Li et al., 2016). Total RNA extraction of jujube fruit was conducted with the method described by Moore et al. (2005). RT-qPCR was performed according to Li et al. (2016). The first-strand cDNA was synthesized with a PrimeScript RT reagent Kit with gDNA Eraser. RT-qPCR analyse was carried out with a Step One Plus Real-Time PCR system with SYBR Premix ExTaq. Primers were designed with Primer Express software 3.0 and the sequences of primers were shown in Supplementary table S.1. PCR conditions were as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The *B. cinerea tubulin* gene and the jujube *actin9* gene were used as internal reference genes, respectively. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.9. Experimental design and statistical analysis

To determine the effect of ε-PL against *B. cinerea* in jujube fruit, we used two batches of fruit. Each batch of fruit was randomly divided into three groups and performed three independent replicates. For each replicate, all fruit were divided into small groups with 15 fruit randomly, wounded at the same depth, inoculated with the same amount of *B. cinerea* spores, and treated with ε-PL at different concentrations (0, 1000, 2000, or 4000 mg L^{-1}). Sarcocarp samples around the infection sites (0.5 cm) of fruit were collected and used to detect gene expression levels.

To explore the effect of ε-PL on mycelial growth of *B. cinerea*, equal amount of *B. cinerea* spores were added into PDA plates with ε-PL at 0, 150, 300, and 450 mg L^{-1}. Three plates of each ε-PL concentration were used, and the experiment was repeated twice.