International Journal of Food Microbiology 278 (2018) 26–35

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Ethyl *p*-coumarate exerts antifungal activity *in vitro* and *in vivo* against fruit *Alternaria alternata via* membrane-targeted mechanism

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ARTICLE INFO

Keywords:
Ethyl *p*-coumarate
Alternaria alternata
Antifungal
Plasma membrane
Jujube fruit

ABSTRACT

The fungus *Alternaria alternata* can cause food contamination by black spot rot and food safety issues due to the production of mycotoxins. In particular, *A. alternata* can infect many fresh fruits and vegetables and lead to considerable postharvest decay during storage and processing. The use of plant-derived products in postharvest disease management may be an acceptable alternative to traditional chemical fungicides. The aim of this study was to assess the antifungal activity of ethyl *p*-coumarate (EpCA) against *Alternaria alternata in vitro* and *in vivo*, and to determine the underlying mechanism. Results indicated that EpCA exhibited pronounced antifungal activity against *in vitro* mycelial growth of *A. alternata*, with half-inhibition concentration of 176.8 μg/mL. Spore germination of the pathogen was inhibited by EpCA in a dose-dependent manner. Moreover, *in vivo* test confirmed that both 100 and 800 μg/mL EpCA significantly reduced disease development of black spot rot in jujube fruit caused by *A. alternata*. The EpCA treatments increased plasma membrane permeability as great leakage of intercellular electrolytes, soluble proteins and sugars of *A. alternata* occurred during incubation. The EpCA treatments also caused increase of the influx of propidium iodide, a fluorescence dye binding nucleus DNA, into the affected spores, indicating the disrupted plasma membrane integrity. Observations of ultrastructure further evidenced the damage to plasma membrane and morphology of *A. alternata* caused by EpCA, which resulted in distortion, sunken and shrivelled of spores and mycelia of the pathogen. In addition, fluorometric assay by confocal laser scanning microscopy confirmed that the EpCA treatments induced endogenous reactive oxygen species (ROS) formation in the spores of *A. alternata*, with stronger and more stable accumulation of ROS at higher concentration of EpCA. Therefore, heavy oxidative damage to cellular membranes and organelles might happen as demonstrated by the severe occurrence of lipid peroxidation of the pathogen treated with EpCA. Taken together, these results indicated that EpCA exerts antifungal activity *via* membrane-targeted mechanism and it would be a promising candidate to control postharvest diseases of fruits.

1. Introduction

Alternaria alternata, a ubiquitous fungus widely distributed in our environment due to its strong adaptability, usually lead to processed food contamination and black spot rot of fresh fruits and vegetables (Estiarte et al., 2017). In addition, *A. alternata* can produce mycotoxins such as tenuazonic acid, alternariol methylether and alternariol, which can accumulate greatly in fruits and the processed food products, leading to food safety issues and threatening the health of human beings (Estiarte et al., 2017; Wang et al., 2017). Infection by *A. alternata* usually results in considerable economic losses of fruits and vegetables after harvest, such as jujubes (Wang et al., 2009; Yan et al., 2015), cherry tomatoes (Pane et al., 2016), apples (Harteveld et al., 2014), citruses (Sanzani et al., 2016) and blueberries (Greco et al., 2012). Currently, synthetic fungicides including iprodione and inorganic copper fungicides have been extensively used as the primary mean of combating the black spot rot (Estiarte et al., 2017). Unfortunately, widespread application of synthetic antifungal agents has led to a conspicuous increase in drug resistance and many of these chemicals are proved to be detrimental to human health and harmful to environment (Wang et al., 2009). Accordingly, it is necessary to develop an environmentally friendly acceptable alternative to these traditional fungicides for controlling black spot rot of fruits and vegetables.

Nowadays, there is an increasing interest in developing food preservatives or antifungal agents for fruit fresh-keeping with plant-derived biologically active compounds due to their extensive

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https://doi.org/10.1016/j.ijfoodmicro.2018.04.024
Received 23 January 2018; Received in revised form 6 April 2018; Accepted 13 April 2018
Available online 19 April 2018
0168-1605/

antimicrobial properties (Tian et al., 2012). Phenolic acids constitute a large proportion of biologically active compounds and exert extensive antifungal effects on various postharvest pathogens such as *A. alternata* (Pane et al., 2016), *Penicillium expansum* (da Rocha Neto et al., 2015) and *Botrytis cinerea* (Ahn et al., 2005). Phenolic acids are found abundant in fruits and vegetables and were commonly identified with chlorogenic acid, gallic acid, ferulic acid, *p*-coumaric acid, caffeic acid, *etc*. (Barbieri et al., 2017). Among them, chlorogenic and gallic acids were found to intensely inhibit the growth of several fungal pathogens including *Alternaria* spp. (Lattanzio et al., 1994). Gallic acid, chlorogenic acid, catechin, caffeic acid, *p*-coumaric acid and ferulic acid in pepper extracts showed *in vitro* and *in vivo* inhibition against *A. alternata* (Pane et al., 2016). In particular, *p*-coumaric acid was demonstrated to inhibit mycotoxin production of *A. alternata* other than its inhibition on the growth of the pathogen (Wang et al., 2017). Furthermore, the phenolic acid methyl as well as alkyl esters have been reported to inhibit bacteria and molds and also to have antioxidant activities in food products (Daayf et al., 1997). The esterified phenolic acids were even suggested to have stronger antifungal activity than that of free phenolic acids (Ahn et al., 2005). For example, methyl gallate exhibited more pronounced antifungal activity than gallic acid against *Magnaporthe grisea* and *Botrytis cinerea* (Ahn et al., 2005). Methyl esters of *p*-coumaric, caffeic and ferulic acids showed higher inhibitory activities than their corresponding acids against *Botrytis cinerea*, *Pythium ultimum* and *Pythium aphanidermatum* (Daayf et al., 2000). Besides, the ester-bound phenolic acids were found to have wider spectrum of antimicrobial activity against bacteria and fungi than free phenolic acids (Ayaz et al., 2008). So far, little information is available on the antifungal activity of *p*-coumaric acid esters.

p-Coumaric acid (4-hydroxycinnamic acid) is a major phenolic acid that mainly exists as esterified or free acid in various fruits such as jujubes (Wang et al., 2010), pears (Salta et al., 2010) and sweet cherries (Wang et al., 2017). *p*-Coumaric acid is of great interest partly due to its antioxidant, anti-inflammatory, anticancer, chemoprotectant, antidiabetic and antimicrobial properties (Amalan et al., 2016; Ota et al., 2011). Furthermore, esterified *p*-coumarates, such as methyl and ethyl *p*-coumarate (EpCA, Fig. 1), were the main derivatives of *p*-coumaric acid found in natural plants (Al-Barham et al., 2016; Choi et al., 2016; Pino et al., 2004). The esterified *p*-coumarates have stronger biological properties on antioxidant and antiradical activity than *p*-coumaric acid (Sharma et al., 2014). Methyl *p*-coumarate has been reported the antifungal activity against *Cladosporium cladosporioides*, *Colletotrichum gloeosporioides*, *Curvularia* sp., *Penicillium* sp., *Helminthosporium* sp. and *A. alternata* (Basha et al., 2016; Daayf et al., 1997). With regard to ethyl *p*-coumarate, which is more hydrophobic and lipophilic than *p*-coumaric acid, the increased lipophilicity was considered to be a structural feature indispensable to antifungal activity (Lattanzio et al., 1994). However, to the best of our knowledge, little on the antifungal property and the mechanism of antifungal action of EpCA is available.

Accordingly, the aim of this work was to investigate the effect of EpCA *in vitro* against the economically significant fungus of *A. alternata*. In addition, changes of metabolism and functional activity related to cell membrane of *A. alternata* induced by EpCA were investigated to explore the possible mechanism. Further study was conducted to evaluate the effect of the EpCA treatment on the *in vivo* growth of *A. alternata* on jujube fruit. The data would provide a theoretical basis for the future control of postharvest black spot rot of fruits and vegetables.

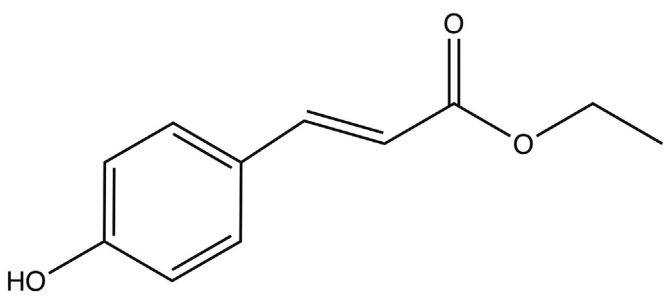

Fig. 1. Structure of ethyl *p*-coumarate (EpCA).

2. Materials and methods

2.1. Chemicals

The biochemical reagent ethyl *p*-coumarate (EpCA, ≥98%) was obtained from Goybio Co., Ltd. (Shanghai, China). EpCA was dissolved in 5% ethanol-sterile distilled water (v/v), and then filtrated through a 0.22-μm microporous membrane to remove bacteria. Propidium iodide (PI), 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and L-cysteine were acquired from Sigma–Aldrich Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Fungal isolate and preparation of spore suspension

The fungal pathogen *Alternaria alternata* (Fr.) Keissler was isolated from an infected jujube fruit and cultured in potato dextrose agar (PDA) at 28 °C for two weeks. The plates were then flooded with sterile distilled water and gently rubbed with a sterile glass spreading rod to release spores. Spore suspensions were filtered through four layers of sterile cheesecloth to remove mycelial fragments, and the spore concentration was adjusted to 1×10^6 spores/mL with the aid of a haemocytometer prior to use.

2.3. Measurement of mycelial growth

The measurement of *in vitro* mycelial growth was conducted according to the method described by Tatsadjieu et al. (2009). An *A. alternata* colony (5 mm in diameter) obtained from a 14-day cultured Petri dish was placed in the center of a plate (90 mm diameter) containing 15 mL of amended PDA with various concentrations of EpCA at 0 (control), 25, 50, 100, 200, 500, 800 and 1000 μg/mL. The plates were sealed with parafilm and incubated for 9 days at 28 °C. The mycelial growth was evaluated each day by measuring the average of two perpendicular radii (mm) of each colony. The predicted values of lag phase (day) and radial growth rate (mm/day) were obtained by applying a linear regression model in radial growth curves. Half-inhibition concentration of EpCA for the fungus was calculated. Each replicate consisted of three plates and three replicates of each treatment were performed.

2.4. Spore germination assay

An aliquot of 100 μL freshly prepared spore suspension (1×10^6 spores/mL) was incubated in 5 mL potato dextrose broth (PDB) medium containing EpCA at the final concentrations of 0 (control), 25, 50, 100, 200, 500, 800 and 1000 μg/mL. An aliquot of 30 μL of the mixture was then transferred to a concave slide which was then placed inside a Petri dish, with a piece of filter paper moistened with sterilized water placed on the bottom to keep moisture. The spore germination was microscopically observed after incubation at 28 °C for 0, 2, 4, 8 and 12 h. The germination was determined when the length of a germ tube exceeded half of the small-end diameter of the spore according to Pane et al. (2016). At least 200 spores were examined in each visual field. Spore germination rate was expressed as a percentage of the germinated spores to the total calculated spores. Each replicate consisted of three observations and three replicates of each treatment were performed.

2.5. Measurement of cellular leakage

The leakage of electrolytes of *A. alternata* was measured according