Industrial Crops & Products 112 (2018) 427–433

Contents lists available at ScienceDirect

Industrial Crops & Products

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

Cinnamaldehyde damaged the cell membrane of *Alternaria alternata* and induced the degradation of mycotoxins *in vivo*

Lingchun Xu, Nengguo Tao, Wenhao Yang, Guoxing Jing*

School of Chemical Engineering, Xiangtan University, Xiangtan, 411105, PR China

ARTICLE INFO

Keywords:
Cinnamaldehyde
Alternaria alternata
Membrane integrity
Membrane permeability
Mycotoxins

ABSTRACT

Alternaria alternata is a widely distributed pathogen that results *Black Rot* disease in fruits and vegetables. This study investigated the effect of cinnamaldehyde against *A. alternata in vitro*, the antifungal activity was closely connected with cinnamaldehyde concentrations and the minimum inhibitory concentration (MIC) was 0.200 μL/mL. Scanning electron microscope (SEM) results showed that cinnamaldehyde altered the morphology of *A. alternata* hyphae, the decrease of total lipids content indicated that the integrity of cell membrane was destroyed after cinnamaldehyde treatment. Furthermore, the release of intracellular components, leakage of electrolyte, and the intracellular presence of propidium iodide staining confirmed that cinnamaldehyde destroyed cell membrane permeability of *A. alternata*. In addition, the mycotoxins synthesized during fungal growth might interact with cinnamaldehyde after membrane destruction, and the concentrations of alternariol (AOH) and alternariol monomethyl ether (AME) were decreased after 0.200 μL/mL cinnamaldehyde incubation for 120 min *in vivo*. In conclusion, cinnamaldehyde was effective to against *A. alternata* by the disruption of plasma membrane, and it could be a safe alternative for fruits and vegetables preservative because of the observed activity in mycotoxins degradation.

1. Introduction

Alternaria alternata has been reported as the most common contaminated fungus in infecting agronomically important crops (Lorenzini and Zapparoli, 2014). Due to rapid growth at room and low temperatures, *A. alternata* are responsible for food spoilage during storage and refrigerated transport (Ostry, 2008). Involved in fruits and vegetables decay, most *Alternaria* species were found to produce a variety of mycotoxins during the growth and resulted potential dangers to human and animal health (Andersen et al., 2015). Recently, chemical fungicides (such as orthophenyl phenate, imazalil, and thiabendazole) were found to be effective in controlling *A. alternata* (Soylu and Kose, 2015), but environmental pollution and fungicide resistance were turned up after long term usage. With the development of alternative methods, effective and ecofriendly strategies will be used for reducing *A. alternata* decay in vegetables and fruits.

Essential oils were suggested as effective and safe approaches for reducing postharvest diseases (Shao et al., 2013; Tao et al., 2014), Soylu and Kose (2015) demonstrated that essential oils derived from oregano (*Origanum onites* L.), thyme (*Thymbra spicata* L.), fennel (*Foeniculum vulgare Mill.*), laurel (*Laurus nobilis* L.), and lavender (*Lavandula stoechas* L. subsp. *stoechas* L.) were effective in against *A. alternata* of citrus. Chen et al. (2014) and Xu et al. (2014) showed that *Cassia* oil, *Citronella* oil, *Laurus* nobilis oil, and *thyme* oil strongly inhibited the growth of *A. alternata in vitro* and *in vivo*, especially cinnamaldehyde in *Cassia* oil, which was most effective in protecting cherry tomato (*Lycopersicon esculentum*) from *A. alternata* infection, but further study about the inhibition mechanism of cinnamaldehyde on *A. alternata* growth was not revealed by the present study.

Several secondary metabolites in *A. alternata*, such as mycotoxins of alternariol (AOH), alternariol monomethyl ether (AME), and tenuazonic acid (TeA), which were generated during fungal growth and resulted potential threat to human health (Prendes et al., 2015). Some studies demonstrated that AOH and AME showed potential genetic toxicity by breaking DNA strand and causing gene mutations in bacterial and mammalian cells (Schwarz et al., 2012), TeA also been found that could inhibit protein biosynthesis on ribosomes and suppress the release of new proteins (Yun et al., 2015). Therefore, inhibiting mycotoxins synthesis and reducing mycotoxins levels are also the main research interests in controlling postharvest decay of vegetables and fruits.

Besides antifungal activity, essential oils also exhibited the activities in inhibiting mycotoxins synthesis (Lappa et al., 2017; Sumalan et al., 2013; Tian et al., 2015). According to Hua et al. (2014),

* Corresponding author.
E-mail address: xing810810@163.com (G. Jing).

https://doi.org/10.1016/j.indcrop.2017.12.038
Received 14 June 2017; Received in revised form 24 November 2017; Accepted 16 December 2017

cinnamaldehyde was the most effective essential oil in against *Aspergillus ochraceus* growth and Ochratoxin A production. Yin et al. (2015) also reported that cinnamaldehyde reduced aflatoxins production by at least 60% in broth culture and chicken feed, the result suggested cinnamaldehyde could be a feed additives to control AF contamination in poultry feed. In addition, cinnamaldehyde also exhibited the activity of degrading mycotoxins *in vivo* and *in vitro*. Sun et al. (2015) reported that 14.8% aflatoxin B_1 in *A. flavus* was degraded after 528.60 mg L^{-1} cinnamaldehyde incubation for 5 days. Xing et al. (2014b) investigated the effect of *cinnamon* oil, citral, *eugenol* oil, *eucalyptus* oil, *anise* oil, and *camphor* oil on the degradation of Fumonisin B_1 *in vitro*, and the result showed that cinnamon oil was the most effective in reducing FB_1 from these tested essential oils.

Extensive literature surveies have proved the antifungal activities of cinnamaldehyde, but no relevant research has found in the relationship between cell membrane integrity and *A. alternata* mycotoxins degradation after cinnamaldehyde treatment. Aside from the inhibition on *A. alternata* growth, the detoxification of cinnamaldehyde on *A. alternata* mycotoxins will be investigated in this study, so the specific contents of this work are: (1) to identify the antifungal activity of cinnamaldehyde on the growth of *A. alternata*; (2) to explore the effects of cinnamaldehyde on plasma membrane integrity of *A. alternata* through observing the surface morphology of hyphae and total lipids content, determining the permeability of the plasma membrane with propidium iodide dyeing, electrolyte leakage and nucleic acid contents; (3) to study the effects of cinnamaldehyde on the degradation of *A. alternata* mycotoxins after membrane damage.

2. Materials and methods

2.1. Fungal species

The toxigenic *A. alternata* strain was isolated from infected table grape fruit (*Vitis vinifera* L. cv. Red Globe) and cultured on potato dextrose agar (PDA) media at 25 °C (Xu et al., 2017).

2.2. Mycotoxins standards and chemicals

AOH and AME were supplied by Pribolab (Pte. Ltd. Singapore) and dissolved in methanol for HPLC analysis. Cinnamaldehyde was purchased from Shanghai Darui Essential Oil Corporation (Shanghai, China), the essential oil was obtained by steam or hydrodistillation of *Cassia* Oil. Cholesterol (95%) and phosphor vanillin (98%) were purchased from TCI Co., Ltd. (Shanghai, China).

2.3. Antifungal activity of cinnamaldehyde

The effect of cinnamaldehyde on the growth of *A. alternata* mycelial *in vitro* was determined by the methods of Tao et al. (2014). Cinnamaldehyde (0.000, 0.013, 0.025, 0.050, 0.100, 0.200, 0.400 μL/mL) were added to 20 mL sterile PDA medium at 40–45 °C and then poured into glass Petri dishes (90 mm in diameter). One mycelial disc (6 mm) from 5-day-old fungal cultures from PDA were transferred on the center of each new Petri plate and incubated at 25 °C for 3 days. Treatments with different concentrations of cinnamaldehyde were carried out in triplicate, respectively. Percentage of mycelial growth inhibition was calculated by the formula:

$$MGI\ (\%) = [(dc\text{-}dt)/dc] \times 100.$$

Where *dc* (mm) is the mean colony diameter for the control sets and *dt* (mm) is the mean colony diameter for the treatment sets. The minimum inhibitory concentration (MIC) was considered as the lowest concentration that no visual hyphal growth was observed after 2 days incubation.

2.4. Mycelium culture and scanning electron microscopy (SEM)

Fungal mycelia were obtained from 3 days cultures cultured in potato dextrose broth (PDB) under rotary shaking condition (150 r/min) at 25 °C, and then treated with cinnamaldehyde at various concentrations (0, 0.5 × MIC and MIC) for 0, 30, 60 and 120 min.

The hyphae morphology of *A. alternata* was measured using scanning electron microscopy (SEM) following the protocols of Zhou et al. (2014) and Yahyazadeh et al. (2008). Mycelia were fixed with 2.5% (v/v) glutaraldehyde in 0.05 mol/L phosphate buffer (pH 6.8) at 4 °C over night, then washed with sterile water for 3 times (each time 20 min). After washing, the samples were dehydrated in an ethanol gradient of 30%, 50%, 70%, 95% for 20 min, and finally treated with pure ethanol for 45 min. The fungal mycelia were placed in the ultra low temperature freezer for 2 h, and then dried in the vacuum freeze for 6–8 h. The dehydrated specimens were coated with a thin layer (20–30 nm) of gold-palladium (40 s, 1.8 mA, 2.4 kV) as conductive medium by a sputter coating procedure (Polaron, Watford, UK). Fungal morphology was observed in a JSM-6610LV SEM (JEOL, Japan) operating at 30 kV at 3000 × level of magnification.

2.5. Lipid contents determination

Total lipids content of *A. alternata* cells were performed by the methods described in Helal et al. (2007). The 3-day-old mycelia from 100 mL PDB was collected and filtered with four layers of sterile gauze, the samples were dried with a vacuum freeze drier for 8 h. About 0.02 g of dry mycelia was homogenized with liquid nitrogen and redissolved in 3 mL distilled water. 0.2 mL of each homogenate was extracted with 3.8 mL of methanol: chloroform: water mixture (2:1:0.8, v/v/v) in a clean dry test tube with vigorous shaking for 10 min. The tubes were centrifuged at 4000*g* for 10 min, after that the lower phase containing lipids were thoroughly mixed with 0.2 mL NaCl. After centrifuged at 4000*g* for 10 min, an aliquot of 0.1 mL chloroform and lipid mixture was transferred to a novel tube, and then heated for 10 min in a boiling water bath after adding 0.2 mL H_2SO_4. Subsequently, three milliliter phosphor vanillin were added and shaked vigorously, and then incubated at room temperature for 10 min. The absorbance at 520 nm was utilized to calculate total lipids content (mg/g dry weight) from the standard calibration curve using cholesterol as a standard.

2.6. Plasma membrane integrity with propidium iodide (PI) dyeing

Membrane integrity of *A. alternata* cells exposed to 0, 0.5 × MIC, and MIC cinnamaldehyde was assessed according to Liu et al. (2010). The 3-day-old mycelia from 100 mL PDB were collected and washed 3 times with phosphate-buffered saline (PBS, 0.05 M, pH 7.2), then 0.5 g mycelia was taken into round-bottom flask (50 mL) and stained with propidium iodide (PI) for 5 min at 30 °C. After filtration, the mycelia were washed thrice with PBS solution (0.05 M, pH 7.2) to remove residual dye, and the mycelia were observed with a fluorescence microscope (Nikon INTENSILIGHT C-HGFI, Nikon ECLIPSE TS100, Japan).

For the measurement of fluorescence intensity, about 0.05 g mycelia were crushed with liquid nitrogen and homogenized with 2 mL PBS buffer (0.05 M, pH 7.2). After centrifuged at 2000*g* for 5 min at 4 °C and the supernatant was analyzed by a fluorophotometer (Lengguang Tech, Shanghai, China) at an excitation wavelength of 546 nm and an emission wavelength of 590 nm.

2.7. Electrolyte leakage and nucleic acid content

Electrolyte leakage and nucleic acid content of *A. alternata* cells were measured according to the methods of Shao et al. (2013) with minor modification. The 3-day-old mycelia from 100 mL PDB were collected and washed 3 times with PBS solution (0.05 M, pH 7.2). After filtration, about 0.3 g mycelium were suspended in a centrifuge tube