Food Chemistry 178 (2015) 76–81

Contents lists available at ScienceDirect

Food Chemistry

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

Citral exerts its antifungal activity against *Penicillium digitatum* by affecting the mitochondrial morphology and function

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ARTICLE INFO

Article history:
Received 20 October 2014
Received in revised form 9 January 2015
Accepted 14 January 2015
Available online 21 January 2015

Keywords:
Citral
ATP
TCA
Mitochondrial membrane permeability
Penicillium digitatum

ABSTRACT

This work investigated the effect of citral on the mitochondrial morphology and function of *Penicillium digitatum*. Citral at concentrations of 2.0 or 4.0 μL/mL strongly damaged mitochondria of test pathogen by causing the loss of matrix and increase of irregular mitochondria. The deformation extent of the mitochondria of *P. digitatum* enhanced with increasing concentrations of citral, as evidenced by a decrease in intracellular ATP content and an increase in extracellular ATP content of *P. digitatum* cells. Oxygen consumption showed that citral resulted in an inhibition in the tricarboxylic acid cycle (TCA) pathway of *P. digitatum* cells, induced a decrease in activities of citrate synthetase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinodehydrogenase and the content of citric acid, while enhancing the activity of malic dehydrogenase in *P. digitatum* cells. Our present results indicated that citral could damage the mitochondrial membrane permeability and disrupt the TCA pathway of *P. digitatum*.

1. Introduction

Green mold of citrus, caused by *Penicillium digitatum*, is the most serious postharvest disease of citrus. Citral, a naturally occurring isoprenoid with two isomers (geranial and neral), reportedly exerts strong anti-fungal activity against *P. digitatum* (Droby et al., 2008; Wolken, Tramper, & van der Werf, 2002; Wuryatmo, Klieber, & Scott, 2003). In our recent papers, citral with minimum inhibitory concentration (MIC, 2.0 μL/mL) and minimum fungicidal concentration (MFC, 4.0 μL/mL) was illustrated to have a good anti-fungal effect against *P. digitatum* in citrus (Fan, Tao, Jia, & He, 2014; Tao, Oyang, & Jia, 2014).

Mitochondria are a type of rod-shaped organelle found in large numbers in most cells. The main function of the mitochondria is to produce the energy currency of the cell, ATP, through tricarboxylic acid cycle (TCA) and oxidative phosphorylation. TCA occurs in the mitochondrial matrix and provides NADH, H^+ and $FADH_2$ to oxidative phosphorylation and therefore serves an extensive regulatory role in ATP yield (Kader & Saltveit, 2003). In addition, mitochondria also play a prominent role in many other metabolic tasks, such as regulation of the membrane potential, apoptosis-programmed cell death, calcium signaling (including calcium-evoked apoptosis), regulation of cellular metabolism, certain haem synthesis reactions (see also porphyrin), and steroid synthesis (Brookes, Yoon, Robotham, Anders, & Sheu, 2004; Gunter, Buntinas, Sparagna, Eliseev, & Gunter, 2000; Loeffler & Kroemer, 2000; Maechler, 2002).

Previously, citral was found to have the capacity for damaging mitochondria by causing morphological changes of some fungi, such as *Aspergillus flavus*, *Trichophyton mentagrophytes*, and *Tagetes patula* (Luo & Jiang, 2002; Nogueira et al., 2010; Park et al., 2009; Romagnoli et al., 2005). Bakkali, Averbeck, Averbeck, and Idaomar (2008) found that eukaryotic cells treated with essential oils suffered a decrease in the intracellular ATP pool and increased extracellular ATP. The intracellular ATP concentration decreased compared with that of the control in sorbic acid against *P. roqueforti* (Liewen & Marth, 1985). Przybylski and Bullerman (1980) reported that exposure of *A. parasiticus* cells to sorbic acid resulted in a decreased level of intracellular ATP and a disproportionate increase in the level of extracellular ATP. Previous studies demonstrated that dill oil could inhibit mitochondrial ATPase activity and result in the inhibition of *A. flavus* growth; the antifungal activity of dill oil resulted from its ability to disrupt the permeability barrier of the plasma membrane and from the mitochondrial dysfunction-induced ROS accumulation. The activity of the mitochondrial dehydrogenase of *A. flavus* exposed to dill oil also decreased. Thus dill oil might affect the mitochondrial function of *A. flavus* (Tian et al., 2012).

Citral could also induce a decrease in the TCA-related enzyme activities of *A. flavus* (Luo & Jiang, 2002). However, the effect of citral on the mitochondrial morphology and function of *P. digitatum* is rather limited. This study aimed to determine

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http://dx.doi.org/10.1016/j.foodchem.2015.01.077
0308-8146/

mitochondria-related targets of citral against *P. digitatum*. The targets were investigated by determining: (i) the morphology of mitochondria using scanning electron microscopy (SEM), (ii) ATP content, (iii) the oxygen consumption of *P. digitatum*, and (iv) intermediate product content and enzyme activities in the TCA pathway.

2. Materials and methods

2.1. Fungal species

P. digitatum was provided by the Department of Biotechnology and Food Engineering, Xiangtan University, Xiangtan, China. All the test strains were preserved on potato dextrose agar (PDA) at 28 ± 2 °C. The spores' concentrations were adjusted to 5×10^5 cfu/mL using a hemocytometer.

2.2. Chemicals

Citral (95%) and ATP sodium (⩾98.5%) were obtained from Sigma–Aldrich (St. Louis, MO). Iodine acetic acid was purchased from Sangon Biotech Co., Ltd. (Shanghai, PR China). SDH and MDH reagent kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The kits for assaying the CS, ICDH, α-KGDH activities and CA content were obtained from Solarbio Beijing (Beijing, China). All chemicals were analytical grade or better.

2.3. Extraction of mitochondria

Mitochondria of *P. digitatum* cells with citral at various concentrations (0, MIC and MFC) were extracted according to the method of Luo and Jiang (2002). The 2-day-old mycelia from 40 mL potato dextrose broth (PDB) were collected and centrifuged at 4000*g* for 15 min. The samples were ground with liquid nitrogen and then fully mixed with extraction medium (20 mmol/L HEPES-Tris, 250 mmol/L mannitol, 10 mmol/L KCl, 5 mmol/L EDTA and 20 mmol/L $MgCl_2$, pH 7.2). After filtering with 120-mesh nylon fabric, the mixture was centrifuged at 4000*g* for 10 min and 12,000*g* for 15 min. Finally, the mitochondria were suspended in extraction medium. All operations of preparation and subsequent extraction were carried out at 4 °C.

2.4. Scanning electron microscopy (SEM)

The collected mitochondria were used for SEM observations (Hashem, 2011; Tao et al., 2014). The mitochondria were promptly placed in vials containing 3.0% (v/v) glutaraldehyde in 0.05 mol/L phosphate buffered saline (pH 6.8) at 4 °C. Samples were kept in this solution for 48 h for fixation and then washed with distilled water three times for 20 min each. Subsequently they were dehydrated in an ethanol series (30%, 50%, 70%, and 95%, v/v), for 20 min in each alcohol dilution and finally with absolute ethanol for 45 min. Samples were then critical point dried in liquid carbon dioxide. Mitochondria were placed in desiccators until further use. Following drying, samples prepared were mounted on standard ½″ SEM stubs using double-stick adhesive tabs and coated with gold–palladium electroplating (60 s, 1.8 mA, 2.4 kV) in a SC7620 sputter coater (Polaron, Watford, UK). All samples were examined in a JEOL JSM-6360LV SEM (JEOL, Tokyo, Japan) operating at 25 kV at 30,000× level of magnification.

2.5. Determination of intracellular and extracellular ATP contents

The intra- and extracellular ATP contents of *P. digitatum* cells with citral at various concentrations (0, MIC and MFC) were determined according to the method of Dai et al. (2008). The mycelia from 40 mL PDB were collected and centrifuged at 4000*g* for 15 min. About 0.1 g of mycelia with 10 mL ultrapure water of the lower layer (intracellular ATP) were taken into a Eppendorf tube and 5 mL extraction medium (2 mmol/L $MgSO_4$, 100 °C) were added to the cell solution. The Eppendorf tubes were maintained at 100 °C for 10 min, cooled in an ice bath, the contents ground with liquid nitrogen and centrifuged for 10 min at 8000*g*. The supernatants were placed in ice immediately to prevent intracellular ATP loss until measurement. The extraction of extracellular ATP (upper layer) used the same method but without the grinding with liquid nitrogen step.

HPLC was conducted on a Shimadzu LC-20AT liquid chromatography system (Shimadzu Scientific Instrument, Kyoto, Japan) equipped with a model LC-20AT solvent delivery system, a model SPD-M20A photo diode array detection system, and an Empower Chromatography Manager. The sample extracts were separated and analyzed using a C_{18} column (250 × 4.6 mm, 5 μm; Shimadzu) at 25 °C. KH_2PO_4–K_2HPO_4 buffer (50 mmol/L, contain 1 mmol/L EDTA, pH 6.0) was used in this experiment. The mobile phase was buffer–methanol (97.5:2.5, v/v) with a 12 min isocratic elution. The flow rate was 1.0 mL/min. Chromatographic peaks were identified by comparing the retention times and spectra against known standards. The detection wavelength was 259 nm. Aliquots of 20 μL were directly injected into the HPLC for the determination. All injections were repeated three times.

2.6. Effect of citral on the respiratory metabolism

Oxygen consumption was measured by a dissolved oxygen meter (JPSJ-605; Leici, Shanghai, China). The effect of citral on the respiratory metabolism of *P. digitatum* was measured according to the method described previously (Guo, Ma, Guo, & Xu, 2005). The fully oxygenated fungal suspension (2.0 mL of 5×10^5 cfu/mL) in phosphate buffer (7.2 mL of pH 7.0) was added to the chamber maintained at room temperature, and allowed to equilibrate for 5 min followed by the addition of 0.8 mL of 1.0% glucose substrate and the system was hermetically sealed. Citral was added to give final concentrations of 2.0 and 4.0 μL/mL after 10 min placement. According to the respiratory rate of the *P. digitatum* before and after citral addition, the respiratory inhibition percent of *P. digitatum* was evaluated using the following equation:

$$I_R(\%) = \frac{(R_0 - R_I)}{R_0} \times 100$$

where I_R represents the respiratory inhibition percent of *P. digitatum* after typical inhibitors (malonic acid, iodine acetic acid and phosphate sodium) or citral addition, R_0 and R_I represent the respiratory rate of *P. digitatum* before and after typical inhibitors or citral addition, respectively.

According to respiratory rate of *P. digitatum* before and after typical inhibitors addition, respiratory superposing inhibition percents of *P. digitatum* were evaluated using the following equation:

$$R_R(\%) = \frac{(R_I - R_I')}{R_I} \times 100$$

In this experiment, typical inhibitors (0.5 mg/mL) were added after citral addition. R_R represents the respiratory superposing inhibition percent of *P. digitatum* after typical inhibitors addition. R_I represents the respiratory rate of *P. digitatum* after citral addition and R_I' represents the respiratory rate of *P. digitatum* after typical inhibitors addition. Each experiment was repeated three times.