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Inhibitory effect of chlorine dioxide (ClO_2) fumigation on growth and patulin production and its mechanism in *Penicillum expansum*

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ABSTRACT

The effect of chlorine dioxide (ClO_2) on *Penicillium expansum* growth and patulin production *in vitro* and on its disease severity in apples was investigated. ClO_2 treatment significantly reduced the lesion diameter in apples infected with *P. expansum* and inhibited mycelial growth and spore germination. After ClO_2 treatment, *P. expansum* mycelial morphology was strongly affected, and a reduced spore plasma membrane integrity with an increased malondialdehyde (MDA) mycelial content was observed. Moreover, patulin production by *P. expansum* was reduced by ClO_2 treatment, both in apples and in PDB meidium. These data showed new and precise clues to the inhibitory effect of ClO_2 on *P. expansum* growth, and provided an effective method to ensure safety of apple products caused by *P. expansum*.

1. Introduction

Studies have shown that nearly 100 different fungi are associated with the decay of fruits and vegetables after harvesting (Xuan & Gu, 2006), *Penicillium expansum* is one of the most destructive pathogens (Jurick et al., 2011), causing blue mold in apples (Yan, Gaskins, Vico, Luo, & Jurick, 2014), peaches (Cao, Yang, Hu, & Zheng, 2011), as well as other fruits, resulting in a huge economic loss to farmers, and producing a significant amount of patulin. Patulin, a secondary fungal metabolite, has extensive and intense toxic effects in humans and animals, and is considered to be teratogenic, carcinogenic, and immunotoxic. In many countries, the patulin content in fruit and processed products is severely restricted (Guo, Zhang, & Li, 2015).

It is important to disinfect fruits and vegetables before storage and processing, in this regard, NaClO is a commonly used disinfectant, which is easy to use and has a low cost. However, it has some disadvantages, for example, NaClO itself is toxic and its bactericidal effect is not long-lived. Moreover, NaClO must be applied by immersing fruit in water, which many types of fruit are unable to bear. Therefore, the most efficient method to treat such fruits is through fumigation or fogging using an appropriate disinfectant to control microorganisms. ClO_2, a strong oxidant, is a Class A1 broad-spectrum, highly efficient, safe chemical disinfectant recognized by the World Health Organization (WHO), as well as the Food and Agriculture Organization of the United Nations (FAO), was used for food preservation (Hwang, Cash, & Zabik, 2003). Previous studies on the actions of ClO_2 have mainly focused on the inhibition of bacterial growth (Lee, Costello, & Kang, 2004; Mahmoud, Bhagat, & Linton, 2007). However, the effects of ClO_2 on fungi, especially with respect to the effect on the postharvest pathogen *P. expansum* and its toxin production, have not been widely studied.

Apple is the fruit that is most susceptible to *P. expansum* infection, and thus to be contaminated with patulin (Beretta, Gaiaschi, Galli, & Restani, 2000). Traditionally, fungicides such as fludioxonil, pyrimethanil, imazalil, TBZ, benomyl, and thiabendazole are the primary methods used to control this fungal disease (Deena, 2014; Neri, Mari, Menniti, Brigati, & Bertolini, 2006). However, the use of these fungicides is becoming restricted owing to an increase in pathogen resistance, and a growing concern for the environment and human health. Based on this, the present study aimed to study the effect of ClO_2 on the growth of *P. expansum*, and investigate the mechanisms involved, as well as explore its ability to control blue mold infection in postharvest apples. The effect of ClO_2 on patulin production was also evaluated. The findings of this study will provide a strong reference for exploring the potential antifungal mechanisms of ClO_2, and explore a new method to control this fungal disease and its production of patulin to ensure the safety of apple products.

2. Materials and methods

2.1. Pathogen culture

P. expansum was provided by the Institute of Botany, the Chinese

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Academy of Sciences, China. It was cultured on potato dextrose agar (PDA) at 25 °C. A suspension of spores was obtained from a 7-day-old culture grown at 25 °C, by flooding the dishes with sterile distilled water, and passing the suspension through two layers of sterile cheesecloth to remove hyphal fragments. The spore suspension concentration was adjusted to 10^6 spore mL^{-1} using a hemocytometer.

2.2. Fruit

Apples (Red Fuji) without any wounds or rotting, were purchased from the local fruit market in the Changqing District in Jinan City of Shandong Province, China. Fruit of uniform size, maturity, and color were washed with a sodium hypochlorite solution (0.5% active chlorine) for 2 min, rinsed twice with tap water, and then air-dried at 25 °C (Guo, Wu, Xiao, & Wang, 2010). The prepared apples were randomly divided into the appropriate experimental groups prior to use.

2.3. Preparation of a solid ClO_2 releasing agent

A ClO_2 tablet (10 mg) (Beijing Golden Pool Scientific Co. Ltd, Beijing, China, 10%) was powdered and mixed evenly with 990 mg of β-cyclodextrin, and the mixture was pressed into tablets (1 g weight), the concentration of ClO_2 in tablets was detected using an iodometric measurement and then used immediately for experiments. The principle of the five-step iodometric method utilizes the degree of I^- reduction of various chlorides as a function of the pH value. Titrate free I_2 with sodium thiosulfate standard solution to distinguish ClO_2, $ClO_2{}^{-1}$, Cl_2, ClO_3. The specific operation steps refer to the method of Tian, Tie, Yang, Ci, and Zhu (2018).

2.4. Antimicrobial effect assays

Firstly, the antimicrobial effects of ClO_2 gas and sodium hypochlorite (NaClO) against *P. expansum* were compared. The inhibitory effect of ClO_2 on the mycelial growth of *P. expansum* was determined according to the method of Lai et al. (2016) with some modifications. A 7 mm-diameter mycelial disk obtained from a 1-day-old culture of *P. expansum* was placed in the center of each petri dish (90 mm-diameter), and the petri dish was placed in a plastic box (10 L) containing different concentrations of ClO_2 (0, 100, 200, 300, and 400 mg L^{-1}), and incubated at 25 °C. After 5 days, the colony diameter was determined using the cross method. Each treatment was performed in quadruplicate, and the experiment was repeated for three times.

The inhibitory effect of NaClO on the mycelial growth of *P. expansum* was determined according to the method of Liu, Zhou, and Fu (2017) with some modifications. Then, approximately 20 mL of PDA containing different concentrations of NaClO (0, 500, 1000, 1500, 1800, and 2000 mg L^{-1}) were poured into 90 mm-diameter Petri dishes, respectively. After that, 7 mm-diameter mycelial disks obtained from a 1-day-old culture of *P. expansum* were placed in the center of each petri dish, and incubated at 25 °C. After 5 days, the colony diameter was determined using the cross method. Each treatment was performed in quadruplicate, and the experiment was repeated for three times.

The effect of ClO_2 on spore germination and germ tube elongation in *P. expansum* was determined according to the method of Liu et al. (2017), with some modifications. A 20 μL of *P. expansum* spore suspension (1×10^6 spores mL^{-1}) was coated onto petri dishes containing 20 mL of PDA, and the petri dishes were placed in a plastic box (10 L) containing different concentrations of ClO_2 (0, 50, 100, 150, and 200 mg L^{-1}), and incubated at 25 °C. The germination rate and germ tube length of the spores were assessed microscopically at 6, 8, 10, 12, and 14 h, respectively. A minimum of 200 spores were observed per treatment. The experiment was repeated for three times.

Mycelial disks (7 mm-diameter) obtained from a 1-day-old culture of *P. expansum* were placed in the center of each petri dish (90 mm-diameter), and the petri dishes were placed in a plastic box (10 L) containing different concentrations of ClO_2 (0, 100, 200, 300, and 400 mg L^{-1}), and incubated at 25 °C. After treatment for 0, 2, 4, 6, and 8 days, the spore yields were determined using the method described by Hou et al. (2014).

The effect of ClO_2 on lesion diameter in apples was determined using the method of Li, Lei, Song, Lai, and Xu (2017), with some modifications. Two wounds (3 mm deep × 3 mm wide) were introduced with a sharp instrument at symmetrical parts on the apple equator, and each wound was inoculated with 20 μL of a spore suspension (1×10^6 spore mL^{-1}). After air-drying for 60 min, the infected fruit were arranged in a packing box (400 mm × 250 mm × 100 mm) containing different concentrations of ClO_2 (0, 50, 100, 150, and 200 mg L^{-1}) with a polyethylene bag, and then stored at 25 °C. The decay index and lesion diameter were calculated on the fifth day. Each treatment was repeated twice, with six fruit per replicate. The lesion diameter was measured on the third day. The disease severity for a single apple was assessed based to the lesion area, as follows: level 0, no decay; level 1, slight decay, (decay covering up to 25% of the apple fruit surface); level 2, decaying ranging from 25% to 50% of the area; level 3, severe decay, (decay on more than 50% of the area). The decay index was calculated using the formula DI = df/ND, where, d is the degree of rot severity on the apple, f is the level number, N is the total number of apples examined, and D is the highest degree of disease severity occurring on the scale.

2.5. Morphological examination of fungal hyphae

Based on the preliminary experiment, when the concentration of ClO_2 reached 400 mg L-1, the mycelial growth of *P. expansum* was completely inhibited, which was not suitable for the investigation under SEM, so we selected the concentrations of 0, 100, 200, 350 mg L^{-1} for ClO_2. Morphological changes in *P. expansum* were observed with a scanning electron microscope (SEM, Quanta 200, FEI, USA). 7 mm-diameter mycelial disks obtained from a 1-day-old culture of *P. expansum* were placed in the center of each petri dish, and the petri dishes were place in a plastic box (10 L) containing different concentrations of ClO_2, and incubated at 25 °C. After 7 days, SEM was performed based on the method described by Zhou, Tao, and Jia (2014).

2.6. Pathogen membrane damage

P. expansum spores were incubated in PDB medium containing 0 or 400 mg L^{-1} ClO_2. After incubation at 25 °C for 8 h, the spores were collected and washed with 50 mmol L^{-1} sodium phosphate buffer (pH 7.0) and stained for 30 min with 10 mg L^{-1} of PI. The stained spores were then collected and washed with 50 mmol L^{-1} sodium phosphate buffer (pH 7.0). The spore suspension was examined using a flow cytometer (BD FACSCalibur, USA). The experiment was repeated twice.

2.7. Mitochondrial membrane potential

The mitochondrial membrane potential was measured using Rhodamine 123. *P. expansum* spores were incubated in PDB medium containing 0 or 400 mg L^{-1} ClO_2. After incubation at 25 °C for 8 h, the spores were collected and suspended in PDB medium at a concentration of 10^6 spore mL^{-1}. Following this, the spores were stained for 5 min with 10 mg L^{-1} Rhodamine 123. The stained spores were collected and washed with PDB medium. The spore suspension was then analyzed using a flow cytometer (BD FACSCalibur, USA). The experiment was repeated for two times.

2.8. Lipid peroxidation

Approximately 20 μL of *P. expansum* spore suspension (10^6 spore