Effects of chlorine dioxide on morphology and ultrastructure of *Fusarium sulphureum* and its virulence to potato tubers

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Abstract: The inhibitory effect and its virulence of chlorine dioxide (ClO_2) against dry rot of potato were investigated. Potatoes were treated by ClO_2, then observed for indoor bioassay, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to observe the morphology and ultrastructure of hyphae, and evaluated the control efficiency of ClO_2 on potato tuber (LK99) dry rot by *F. sulphureum* pre-treatment. The results showed that the pathogen of potato dry rot was sensitive to ClO_2, the virulence of regression of $y=5.05+7.308x$, EC_{50} and EC_{90} were 0.3490 and 0.6261 respectively, the treatment of ClO_2 could significantly inhibit the spore germination and mycelium growth of *F. sulphureum,* which was in a concentration-dependent manner, SEM and TEM observed that the morphology and ultrastructure of *F. sulphureum* hyphae were regularly damaged by ClO_2, in vivo experiment further indicated that ClO_2 could effectively control the dry rot of potato tubers with *F. sulphureum*, and ClO_2 at the concentration of 0.75 *u*g/mL could significantly reduce the incidence of potato tuber dry rot and lesion expansion rate. The study showed that ClO_2 could greatly against the pathogen of *F. sulphureum,* which could provide a scientific theoretical basis for the safe and efficient application of ClO_2 in the prevention and control of potato diseases after harvest.

Keywords: potato, dry rot, chlorine dioxide, *Fusarium sulphureum*, virulence, morphology and ultrastructure
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1 Introduction

Potato is widely cultivated throughout the world, there are more than 160 countries to plant potatoes[1]. With greater emphasis on food security in recent years, the planting area and yield of potatoes appear an increasing trend, which has become one of the main economic crops in adjusting the planting structure of China, elevating agriculture efficiency and raising farmers' income[2]. But at least 20%-25% of potatoes are rotted because of pathology and physiological factors after harvest in China, which cause an extremely serious economic loss every year[3]. Dry rot of potato caused by *Fusarium* is the most common in the store, account for more than 50% of fungal diseases in the store. And *Fusarium sulphureum* is the main pathogens brought dry

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rot of potato in the northwest of China[1]. Reducing the trauma during harvest, storage and transportation, or storing potatoes in the environment are in favor of healing of trauma to control the infection of pathogens, all of which could prevent the dry rot of potato tuber. Normally, the chemical synthetic fungicides thiabendazole after the harvest is mainly used. However, the resistance to thiabendazole is generally increased, such as *Fusarium sambucinum*, moreover, the problems of pesticide residues and environmental pollution are widely concerned[4]. In recent years, some natural and generally recognized as safe (GRAS) chemical compounds, such as chitosan[5], nitrite[6], borate[7], sodium silicate[8], β-aminobutyric acid[9], K_2HPO_4[10] and citric acid[11], can effectively control the dry rot of potato tuber, but most of these chemical compounds are in the laboratory research stage, and difficult to apply in production due to the cost. Therefore, it is important to develop the new type of preservatives of low poison, low cost and high efficiency, to prevent the dry rot in storage. As a strong oxidant, ClO_2 is an A1 level broad-spectrum, which is efficient and safe disinfectant of sterilization and preservation that recommended worldwide by WHO and FAO, and also be approved by FDA as the recognized food preservative[12]. ClO_2 could be rapidly adsorbed on the surface of dangerous organisms, effectively restrain pathogens and reduce the rot of vegetables and fruits. At the same time, no harmful substance and odor residue be produced during the sterilization process of ClO_2 application, and it can stay the original flavors of processed fruits and vegetables without negative influence on the food flavors and appearance quality. Thus, ClO_2 is internationally recognized as the excellent performance and much better effected food preservative[13]. In addition, ClO_2 can effectively prevent methionine to synthetizing ethylene in fruits and vegetables, and destroy the synthetic ethylene, to delay fruit senescence and long-term fresh fruits and vegetables[14]. At present, most researches about ClO_2 disinfection is still concentrated on drinking water and microorganisms of food surface. However, virulence and control function of ClO_2 treatment on pathogens after harvest of fruits and vegetables, especially on potato pathogen are rarely reported. Therefore, this article mainly studied the virulence and control function of ClO_2 treatment on the dry rot of potato tuber after harvest through experiment in vivo and vitro, which would provide the theoretical basis for further inhibitory of ClO_2 on dry rot of potato tuber after harvest.

2 Materials and methods

2.1 Potato tubers and chemicals

Potato (*Solanum tuberosum* cv. LK99) was harvested from Huichuan Test Station, Weiyuan County of Gansu Province of China. The potato tubers of uniform size and absence of physical injuries were packed in net bags (15 kg/bag) and immediately transported to the laboratory within 24 h where they were sorted at 3°C-5°C and 80% RH. Before treatment, tubers were surfaced-disinfected with 2% sodium hypochlorite for 2 min, and then rinsed with tap water and air-dried.

Chlorine Dioxide (ClO_2) was purchased from Tianjin zhangda technology development co. (Tianjin, China).

2.2 Pathogen

The fungal pathogen, *F. sulphureum Schlechlendahl*, was provided by Institute of Plant Protection, Gansu Academy of Agricultural Science and Technology. Fungal spores were removed from seven-day-old potato dextrose agar (PDA), and suspended in 5 mL of sterile distilled water containing 0.05% (v/v) Tween-80. The suspensions were filtered through 4 layers of sterile cheesecloth in order to remove adhering mycelia and the concentration was adjusted to 1×10^7 spores/mL using a hemocytometer.

2.3 Inhibitory effect of ClO_2 on *F. sulphureum* in vitro

2.3.1 Indoor bioassay

In accordance with agar-injection method[15]. The sterilization of the agar medium while the liquid poured into the drying and sterilization of 90 mm diameter dish, 30 mL per dish, for medium after cooling, using micropipettor with spore concentration of 1×10^7 spores/mL bacterial suspension 100 μL after coating plate, then with a sterile metal punch (d = 5 mm) into the hole diameter 5 mm, use a toothpick to remove aseptic hole agar after 1-2 drops of 1% agar liquid seal the bottom of the hole and in the hole, etc. after condensation with

different concentrations (0 μg/mL, 0.25 μg/mL, 0.5 μg/mL, 0.75 μg/mL, 1.0 μg/mL and 1.25 μg/mL) of preservatives in each hole, immediately sealed with sealing membrane, each treatment was replicated three times, and with sterile water as control. These operations were performed under sterile conditions. Then incubated at 25°C, the mycelial growth was determined by measuring the colony diameter 5-7 d after inoculation.

2.3.2 Spore germination assay

The effects of ClO_2 on *F. sulphureum* spore germination were assayed according to Li et al.[16]. Briefly, 15 μL aliquots of conidial suspensions (1×10^7 spores/mL) were plated on 1% agar flake (10 mm in diameter) mounted with different concentrations (0 μg/mL, 0.5 μg/mL, 0.75 μg/mL and 1.0 μg/mL) of ClO_2 (12% ClO_2), and then incubated in Petri dishes at 25°C. After 8 h of incubation, germination rates were assessed by observing approximately 200 spores per treatment replicate under a light microscope. Each treatment was replicated 3 times and the experiment was repeated twice.

2.3.3 Mycelial growth assay

The effects of ClO_2 on *F. sulphureum* mycelial growth were assayed according to Yao and Tian[17]. The mycelial disks (10 mm in diameter) from seven-day-old fungal cultures were placed in the center of the Petri dishes (90 mm in diameter) with 30 mL of PDA containing different concentrations of ClO_2 (0 μg/mL, 0.5 μg/mL, 0.75 μg/mL and 1.0 μg/mL), and then incubated at 25°C. The mycelial growth was determined by measuring colony diameter seven-day-old after inoculation. Each treatment was replicated 3 times and the experiment was repeated twice.

2.4 Scanning electron microscopy (SEM) observation

Hypha samples excised from seven-day-old cultures of the fungi treated with different concentrations (0 μg/mL, 0.5 μg/mL, 0.75 μg/mL, 1.0 μg/mL) of ClO_2 were vapor-fixed with 2% (w/v) aqueous osmium tetroxide in a Polaron E500 sputter coater (Polaron, Cambridge, England), and then kept in a dessicator for 2 h at 4°C, air-dried and sputter-coated with gold palladium until examination with a Cambridge Stereoscan 5-150 SEM (LEO Electron Microscopy Ltd., Cambridge, England) operating at 20 kV. Micrographs were taken with a CCD-Camera (America Gatan Company). The experiment was repeated three times on two replicate plates for each treatment and, for each replicate, 10 agar blocks were examined using scanning electron microscopy.

2.5 Transmission electron microscopy (TEM) observation

According to the method of Benhamou et al.[18], mycelial samples (2 mm) were excised from seven-day-old PDA cultures embedded with different concentrations (0 μg/mL, 0.5 μg/mL, 0.75 μg/mL and 1.0 μg/mL) of ClO_2, and then immersed in 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h at room temperature. Samples were subsequently postfixed with 1% (w/v) osmium tetroxide in the same buffer for 1 h at 4°C and dehydrated in Epon812. Sections (0.7 μm) were cut from the Epon-embedded material using glass knives, mounted on glass slides and stained with 1% aqueous toluidine blue prior to examination with a Zeiss Axioscope microscope (Carl Zeiss, Inc., thornwood, NY) primarily for viewing the growth of fungal hyphae. Ultrathin sections (0.1 μm) were collected on formvarcoated nickel grids and were contrasted with either uranyl acetate or lead citrate for direct examination under an electron microscope (JEM-1230, Japan) operating at 80 KV. Micrographs were taken with a CCD-Camera (America Gatan Company). Five samples from each sampling time were examined using five sections per sample.

2.6 Effects of ClO_2 on dry rot of potato tubers

ClO_2 treatments at (0 μg/mL, 0.5 μg/mL, 0.75 μg/mL and 1.0 μg/mL), were also applied to in vivo experiments. The tubers were wounded (5 mm deep and 3 mm wide) with a sterile nail at the equator, then, 10 μL of a conidial suspension of *F. sulphureum* at 1×10^7 spores/mL were added to each wound. Two hours after inoculation, tubers were dipped with ClO_2 at different concentrations with sterile distilled water as the control. Treated tubers were put in 200 mm ×130 mm ×50 mm plastic boxes with sterile water in open petri dish to maintain a high relative humidity, then stored at room temperature (20°C-22°C) and low temperature (4°C-5°C). The incidence of decay at room temperature was determined 15 d after treatment

and 45 d at low temperature. Each treatment contained 3 replicates with 12 tubers per replicate and the experiment was repeated twice.

2.7 Statistical analysis

Experiments were arranged in a completely randomized design, and each treatment was comprised of three replicates. Dates were tested by the analysis of variance using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). Least significant difference (LSDs) was calculated to compare significant effects at the 5% level.

3 Results and analysis

3.1 Inhibitory effect of ClO_2 on growth of dry rot pathogens of potato and indoor toxicity

In Table 1, the growth of *F. sulphureum* was strongly inhibited by ClO_2 in a concentration-dependent manner. Growth of dry rot pathogens was significantly inhibited with the increasing concentration in the process, and complete inhibition at 1.25 μg/mL. As shown in Figure 1, rapidly grown colonies with smooth and forward mycelium were seen in control, but weak expansion in ClO_2 treatments due to inhibition effect.

It can be seen from Table 2 qualitative data probability analysis in indoor bioassay that dry rot pathogen of potato is sensitive to ClO_2 with EC_{50} and EC_{90} value of 0.3490 μg/mL and 0.6261 μg/mL respectively. The chi square value of fitting degree and its significant level values were 5.36 and 0.150239 respectively (p<0.05), greater than 0.05. This indicated that the toxicity regression curve and the established model are reasonable.

Table 1 Inhibition effect of ClO_2 on mycelial growth of dry rot pathogen of potato

Chemical treatment	Concentration /μg·mL^{-1}	Antibacterial circle diameter /mm	Relative inhibition rate/%
ClO_2	0.25	30.0±0.5	14.33cb
	0.50	54.6±0.3	45.92c
	0.75	61.0±0.0	58.69b
	1.00	70.3±1.1	78.32b
	1.25	80.0±0.0	100.0a
CK	0.00	0.0±0.0	0.00e

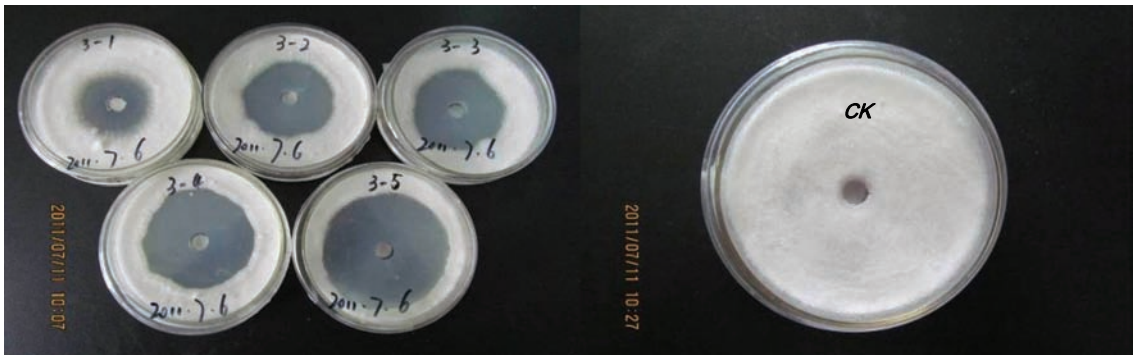


Figure 1 Toxic effect of ClO_2 on dry rot in indoor bioassay

Table 2 Indoor bioassay of ClO_2 to pathogens of potato dry rot

Chemical	Regression equation	EC_{50} /μg·mL^{-1}	EC_{90} /μg·mL^{-1}	Correlation coefficient	chi-square value	95% confidence level of EC50 value	95% confidence level of EC_{90} value
ClO_2	y=5.05+7.308x	0.3490	0.6261	0.9863	5.36	0.3225-0.3778	0.5345-0.7334

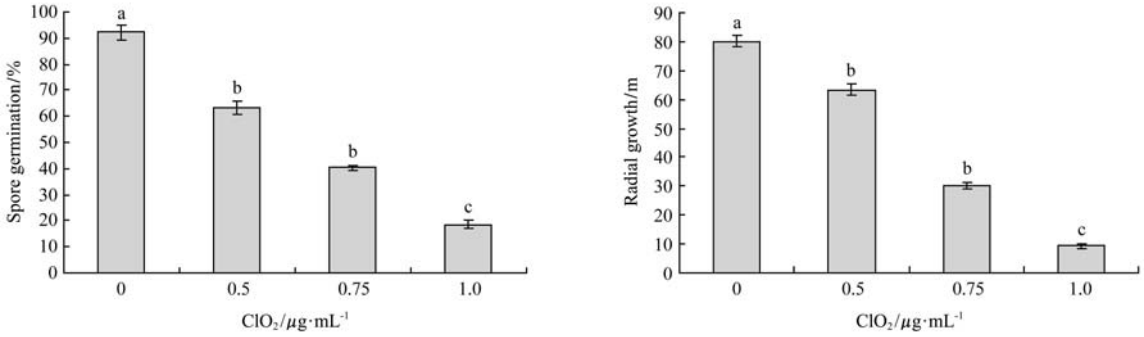
3.2 Effects of ClO_2 treatment on spore germination and mycelial growth of *F. sulphureum*

Generally, the percentage of spore germination increased gradually with incubation time. Nearly all the spores in control without ClO_2 addition were germinated after 8 h incubation (up to 91.6%), however, only 18% of germination at 1.0 μg/mL of ClO_2 were germinated (Figure 2a). The results indicated that the spore germination of *F. sulphureum* was significantly inhibited by different ClO_2 concentrations (p<0.05), and showed obvious concentration effect. In addition, the mycelial growth of *F. sulphureum* was also suppressed by ClO_2 (Figure 2b). At the most effective concentration of ClO_2, 1.0 μg/mL of the mycelial diameter was 87.5% smaller than the control.

3.3 Changes in the hyphal morphology of *F. sulphureum*

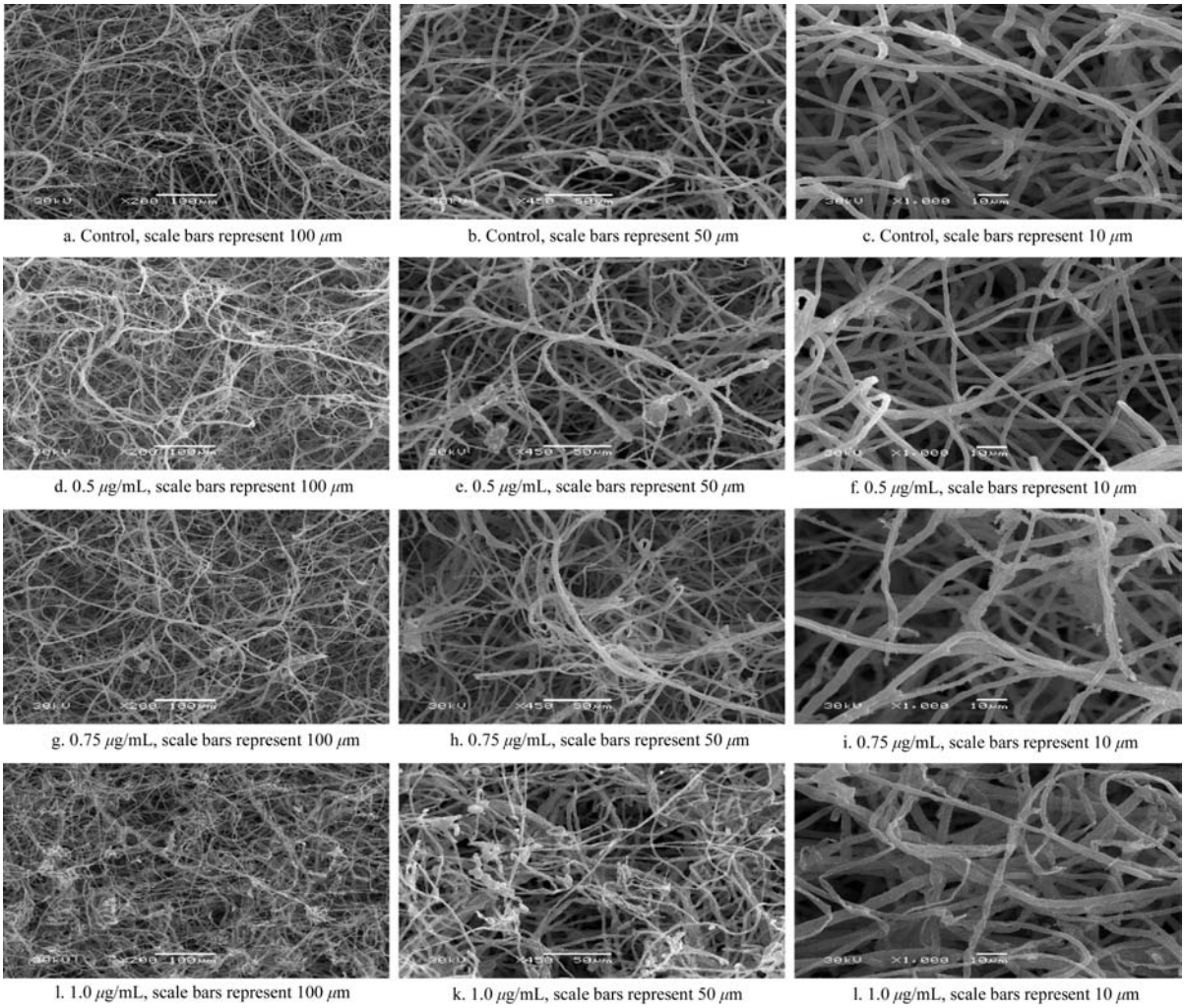
The morphology of *F. sulphureum* hyphae of ClO_2 treatments with different concentrations were shown in Figure 3. By scanning electron micrographs (SEM)

observation, hyphae of the control treatments were long, dense, uniform and round with a smooth surface (Figures 3a-3c). Whereas the growth of hyphae with ClO_2 addition was strongly inhibited, as indicated by mycelial sparsity, asymmetry, curling, and twisting in Figures 3d, 3j and 3g. Newly developed hyphae were thinner and distorted with rough surfaces (Figures 3e, 3h and 3k). The hyphae were swollen and inflated at the brim and mycelium became blasted, wizened, and cupped (Figures 3f, 3i and 3l). With the increasing concentration of ClO_2, the hyphae appeared more winding at 0.75 μg/mL and 1.0 μg/mL of ClO_2 than in control and 0.5 μg/mL, it demonstrated that ClO_2 evidently caused hyphae deformity. Furthermore, the edges of hyphae appeared irregular and swollen, while the hyphae branches were increased and collapsed in the present study.



a b

Figure 2 Effects of different ClO_2 concentrations on spore germination (a) 8 h and mycelial growth (b) 7 d of *F. sulphureum* after incubation at 25°C



a. Control, scale bars represent 100 μm b. Control, scale bars represent 50 μm c. Control, scale bars represent 10 μm

d. 0.5 μg/mL, scale bars represent 100 μm e. 0.5 μg/mL, scale bars represent 50 μm f. 0.5 μg/mL, scale bars represent 10 μm

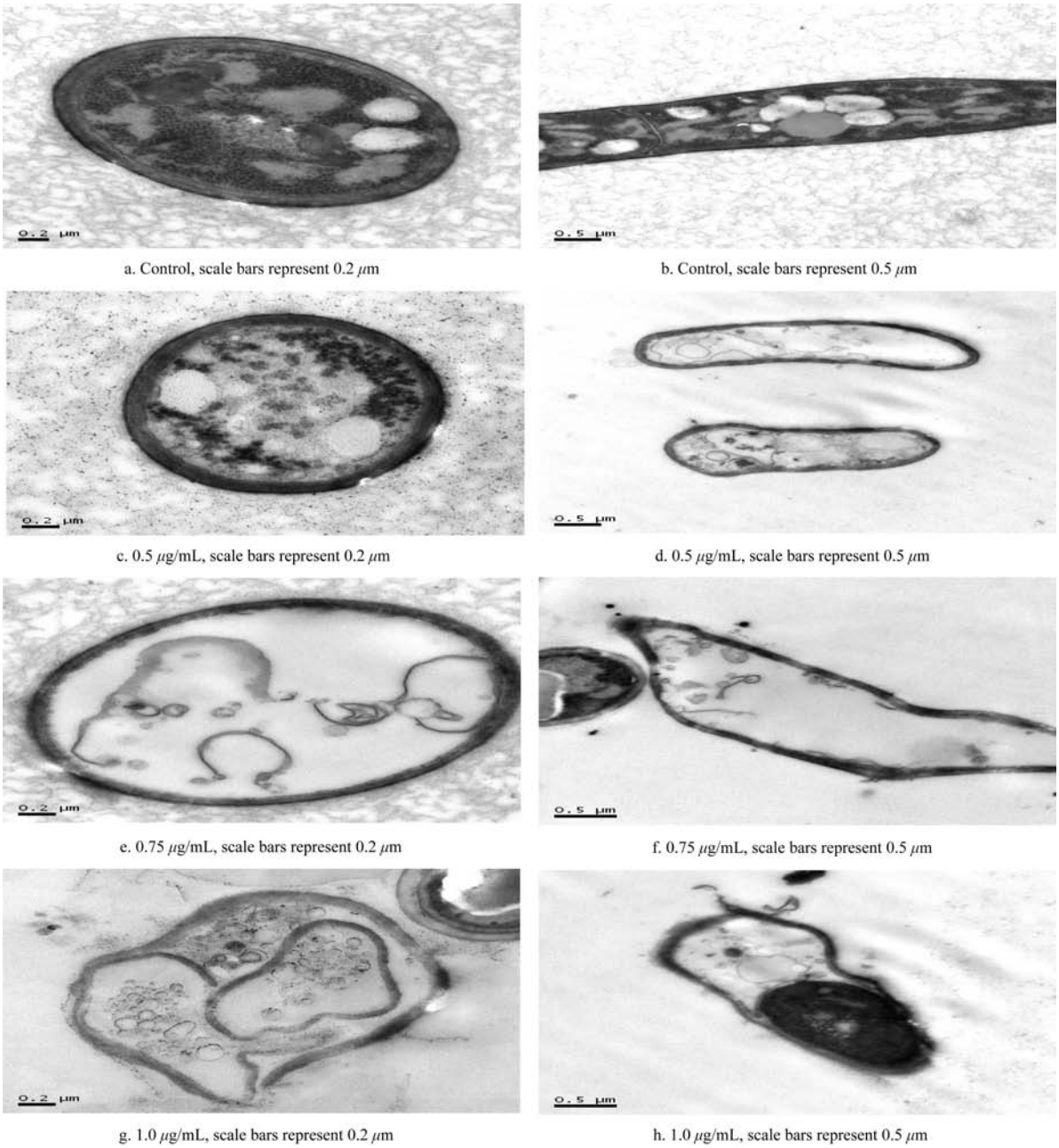
g. 0.75 μg/mL, scale bars represent 100 μm h. 0.75 μg/mL, scale bars represent 50 μm i. 0.75 μg/mL, scale bars represent 10 μm

l. 1.0 μg/mL, scale bars represent 100 μm k. 1.0 μg/mL, scale bars represent 50 μm l. 1.0 μg/mL, scale bars represent 10 μm

Figure 3 Scanning electron micrographs of the hyphae of *F.sulphureum* grown for 7 d on PDA plates containing by different ClO_2 concentrations (0 μg/mL, 0.5 μg/mL, 0.75 μg/mL, 1.0 μg/mL)

3.4 Changes in the hyphal ultrastructure of *F. sulphureum*

The ultrastructure of *F.sulphureum* in control and ClO_2 treatments were shown in Figure 4. By transmission electron microscopy (TEM) observation, normally distributed cytoplasm and vacuoles cell were observed in control, and the hyphal cell wall was thin and uniform (Figures 4a and 4b). In Figures 4c and 4d, the cell wall of 0.5 μg/mL ClO_2 treatment was irregularly thickened, and the daughter hyphae which were inside the collapsed hyphal cells were clearly detected. Besides, some of the daughter hyphae produced the new one, with even cytoplasm, electron-dense material, or cavities. However, abnormally contracted and distorted cells were also found (Figures 4c and 4d). In 0.75 μg/mL and 1.0 μg/mL ClO_2 treatments (Figures 4e, 4f, 4g and 4h), cell walls were obvious thickening, with electron-dense material in the cytoplasm and notably increasing distorted or unshaped hyphae. Electron-dense material in the cytoplasm and cavity were also observed in the longitudinal section of hyphae, but septa of ClO_2 treated hyphae were uniform. In generally, the damage degree of hyphae was evidently related to ClO_2 concentration.



a. Control, scale bars represent 0.2 μm

b. Control, scale bars represent 0.5 μm

c. 0.5 μg/mL, scale bars represent 0.2 μm

d. 0.5 μg/mL, scale bars represent 0.5 μm

e. 0.75 μg/mL, scale bars represent 0.2 μm

f. 0.75 μg/mL, scale bars represent 0.5 μm

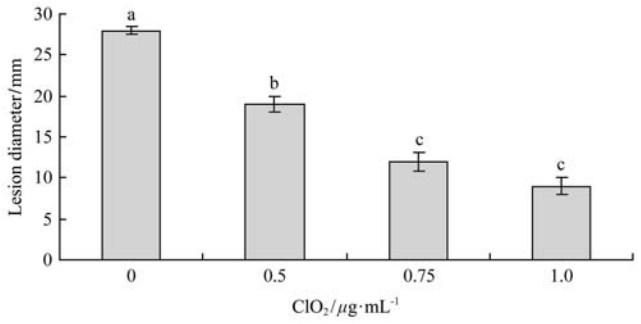
g. 1.0 μg/mL, scale bars represent 0.2 μm

h. 1.0 μg/mL, scale bars represent 0.5 μm

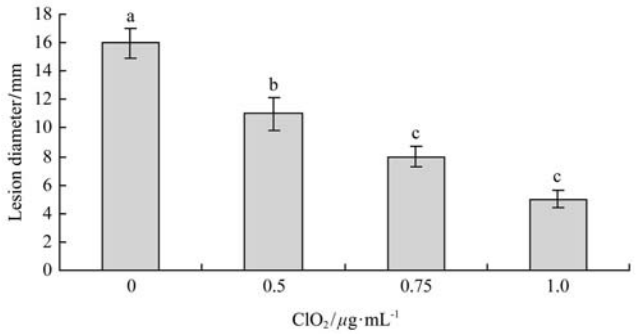
Figure 4 Transmission electron micrographs of the hyphae of *F.sulphureum* grown for 7 d on PDA plates containing by different ClO_2 concentrations (0 μg/mL, 0.5 μg/mL, 0.75 μg/mL and 1.0 μg/mL)

3.5 Effects of ClO_2 on dry rot of potato tubers

To evaluate the curative effect of ClO_2 against dry rot, potato tubers were artificial inoculated with *F. sulphureum,* and then dipped in different concentrations of ClO_2 solution (Figure 5). The results showed that ClO_2 could significantly (p<0.05) reduce the dry rot development in potato tubers both at room temperature and low temperature. ClO_2 effectiveness increased up to the most effective concentration of 1.0 μg/mL at low temperature, where the lesion diameter of dry rot was only 4.93 mm. In addition, the ability of ClO_2 to control dry rot was enhanced when ClO_2 concentration was increased from 0.5 μg/mL to 1.0 μg/mL. However, no significant differences were found between 0.75 μg/mL and 1.0 μg/mL ClO_2 treatments.



a



b

Figure 5 Effects of different ClO_2 concentrations on lesion diameter of dry rot caused by *F. sulphureum* in inoculated potato tuber stored 15 d at room temperature 20°C-22°C (a) and 45 d at low temperature 4°C-5°C (b)

4 Discussion

ClO_2 has been classified as A1 disinfection product by WHO and directly or indirectly used in food industries. It can effectively kill most microorganisms, including bacteria, fungi, algae, viruses, parasites, and is a high effective disinfectant internationally recognized[19-24]. The sturdy results provided conclusive evidence that postharvest treatment with ClO_2 was effective for inhibiting pathogen growth as well as controlling dry rot in inoculated tubers, thereby decreasing the incidence of decay and maintaining tuber quality.

In this study, ClO_2 directly inhibited the spore germination and mycelial growth of *F. sulphureum* in vitro (Figures 1 and 2). The result was consistent with the observation of Li et al.[25] and Hu et al.[6], who found that 100 mM sodium silicate or 0.5 g/100 mL to 1 g/100 mL NO completely inhibited the mycelial growth of *F.sulphureum Schlechlendahl*, which are causative agent of *Fusarium* in potato, respectively. Similar results were also in accord with Chen et al.[27], that 7 mg/L ClO_2 effectively reduced the amount of *S.cerevisae* and *F. tricinctum* spores of 15 min treatment, respectively reduced by 5 lg CFU and 4.6 lg CFU. This indicated that ClO_2 was fungistatic rather than fungicidal against *F. sulphureum.*

The antifungal mechanism of ClO_2 was investigated using SEM and TEM. SEM observations showed that ClO_2 could induced morphological changes such as twisted, wizened and irregular mycelium (Figure 3). Ultrastructural studies by TEM also indicated ClO_2 caused fungal hyphal cells to be seriously damaged, including abnormal thickened cell wall, uneven cytoplasm and malformed hyphae (Figure 4). These results suggest two mechanisms whereby ClO_2 may inhibit decay. Firstly, ClO_2 has a toxicological outcome acting directly at the surface of the fungus, thus damaging the cell wall or membrane. This type of antifungal effect has been seen with boron and its ability to disrupt the cellmembrane of the *B. cinerea*, eventually leading to the leakage of cytoplasmic materials and death of the pathogen[26]. Secondly, ClO_2 can act on the fungus at the intracellular level, by destroying its internal organelles or possibly interfering with the normal metabolism. This is in agreement with research by Lai et al.[27] But this assumption needs further in-depth study.

Although no previous studies on changes in pathogen morphology and ultrastructure after ClO_2 treatment have been undertaken, similar results were reported regarding the antifungal activities of NO, tebuconazole and

chitosan[6,28,16]. The main fungistatic effects of ClO_2 on *F. sulphureum* were changes to the structure of hyphal cells. Several reports indicated that ClO_2 effectively control postharvest rots during storage, delay the onset of infection, and slow down the infection process. In general, the rots are well controlled with increasing ClO_2 concentration. ClO_2 application has been effective against postharvest diseases such as anthracnosis of Ficus carica Linn[29], late bright, soft rot, silve scurf and bacterial ring rot of potato, and that ClO_2 is currently the only marker for late blight control[30]. The results of the present experiment showed that ClO_2 at 0.75 μg/mL was the most effective concentration in reducing decay, while a higher concentration (1.0 μg/mL) did not significantly increase disease control. This may be due to differences in the sensitivity of pathogen species to ClO_2. However, the detailed mechanism for the preventive effect of ClO_2 in our study needs further research.

5 Conclusions

Potato dry rot pathogen is sensitive to ClO_2, spore germination and mycelium growth of *F. sulphureum* were strongly inhibited by ClO_2 in a concentration-dependent manner, and clony morphology and ultrastructure of hyphae were damaged to some extent of dry rot pathogens of potato by ClO_2.

ClO_2 could effectively control the growth of *F. sulphureum*, cures and prevents dry rot of potato tubers, and ClO_2 at the concentration of 0.75 μg/mL could significantly reduce the incidence of potato tuber dry rot and lesion expansion rate.

This study showed that ClO_2 has good antibacterial effect to the pathogen of potato dry rot *F.sulphureum*, ClO_2 could be used as a potential green fungicide, partially replace chemical synthetic fungicides, with a view to better control the disease of potatoes after harvest.

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