

In Vitro and *In Vivo* Inhibitory Effects of Gaseous Chlorine Dioxide Against *Diaporthe batatas* Isolated from Stored Sweetpotato

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Chlorine dioxide (ClO₂) can be used as an alternative disinfectant for controlling fungal contamination during postharvest storage. In this study, we tested the *in vitro* and *in vivo* inhibitory effects of gaseous ClO₂ against *Diaporthe batatas* SP-d1, the causal agent of sweetpotato dry rot. In *in vitro* tests, spore suspensions of SP-d1 spread on acidified potato dextrose agar were treated with various ClO₂ concentrations (1-20 ppm) for 0-60 min. Fungal growth was significantly inhibited at 1 ppm of ClO₂ treatment for 30 min, and completely inhibited at 20 ppm. In *in vivo* tests, spore suspensions were drop-inoculated onto sweetpotato slices, followed by ClO₂ treatment with different concentrations and durations. Lesion diameters were not significantly different between the tested ClO₂ concentrations; however, lesion diameters significantly decreased upon increasing the exposure time. Similarly, fungal populations decreased at the tested ClO₂ concentrations over time. However, the sliced tissue itself hardened after 60-min ClO₂ treatments, especially at 20 ppm of ClO₂. When sweetpotato roots were dip-inoculated in spore suspensions for 10 min prior to treatment with 20 and 40 ppm of ClO₂ for 0-60 min, fungal populations decreased with increasing ClO₂ concentrations. Taken together, these results showed that gaseous ClO₂ could significantly

inhibit *D. batatas* growth and dry rot development in sweetpotato. Overall, gaseous ClO₂ could be used to control this fungal disease during the postharvest storage of sweetpotato.

Keywords : chlorine dioxide, *Diaporthe batatas*, dry rot, sweetpotato

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Sweetpotato (*Ipomoea batatas* Lam.) is one of the most important food crops worldwide, especially in developing countries. The crop currently ranks seventh among staple food crops after rice, wheat, potatoes, maize, barley, and cassava. (Sanusi et al., 2016). The world production of sweetpotato was estimated at 105,190 Mt, in which 74.7% was produced in China and other Asian countries, according to the Food and Agriculture Organization of the United Nations in 2016 (<http://www.fao.org/faostat/en/#data/QC/>). Recently, the consumption of sweetpotato as a healthy food has been increasing; however, postharvest diseases of the crop are among the significant limitations for its production (estimated loss, 15-65%), in terms of both quantity and quality (Ray and Ravi, 2005). Fungal contamination is one of the most serious problems during sweetpotato storage (Ray and Ravi, 2005; Scruggs and Quesada-Ocampo, 2016). Recently, Chakraborty et al. (2017) reported that *Botryodiplodia theobromae*, *Certocystis fimbriata*, *Fusarium* spp., and *Rhizopus oryzae* are the most significant fungi to sweetpotato storage contamination. These fungal pathogens cause local discoloration and disruption of surrounding tissues in infected sweetpotato, resulting in changes involving deterioration of texture and flavor (Amienyo and Ataga, 2007). Because sweetpotato stored in enclosed

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spaces, fungal contamination may spread throughout the storage facility, resulting in significantly contaminated sweetpotato (Wu and Rioux, 2010). Therefore, it is important to apply appropriate measures to limit fungal infection of sweetpotato during storage as described in other crops (Mannaa and Kim, 2018). Fungicides, such as thiabendazole, benomyl, and iprodione, are generally used to control fungal contamination in sweetpotato (Afeke et al., 1998; Singh and Sharma, 2018). However, postharvest application of fungicides is generally avoided because sweetpotato roots are directly used as a food source.

Chlorine has long been used as an effective chemical to control postharvest diseases of fruits and vegetables (Zoffoli et al., 1999). This chemical can react with many organic nitrogen compounds, unsaturated organic compounds, and phenols. Thus, microorganisms can be destroyed through the oxidative action of chlorine on cellular constituents, and in part *via* direct combinations of the compound with membrane proteins and enzymes (Tweddell et al., 2003). In this regard, chlorine dioxide (ClO_2) gas has been shown to effectively reduce food-borne pathogens in many storage crops such as apples, green peppers, lettuce, tomatoes, cabbage, carrots, and peaches, as well as blueberries, raspberries, and strawberries (Du et al., 2002; Han et al., 2001, 2004; Lee et al., 2004; Sy et al., 2005a, 2005b). Thus, ClO_2 gas can be used as an alternative disinfectant to control fungal contamination. Moreover, ClO_2 gas has low toxicity in humans, reacts rapidly, and is effective at low concentrations (Vaid et al., 2010; Wang et al., 2016). ClO_2 gas has been approved for use as a sanitizer for agricultural, industrial, commercial, and medical uses by the U.S. Environmental Protection Agency (2006). Although both aqueous and gaseous ClO_2 are effective as sanitizing agents, gaseous ClO_2 is more effective than aqueous ClO_2 because the gaseous form can penetrate small spaces that the liquid form cannot reach (Han et al., 2001; Lee et al., 2004).

Recently, sweetpotato dry rot caused by *D. batatas* in Korea was reported by Lee et al. (2016). This was the first report of the disease on sweetpotato in the world besides in the USA, where it was first described (Harter and Field, 1912). The disease may, therefore, cause significant reduction in sweetpotato production worldwide, including in Korea. Although ClO_2 gas may inhibit the activity of various deleterious storage fungi, *D. batatas* as an emerging fungal pathogen of sweetpotato was examined in this study. Thus, the objectives of this study were to evaluate the *in vitro* and *in vivo* inhibitory effects of various concentrations and treatment times of gaseous ClO_2 against growth of *D. batatas* and dry rot development in sweetpotato.

D. batatas SP-d1, isolated from a diseased sweetpotato

root (cv. Juwhangmi) and supplied by the Bioenergy Crop Research Center, National Institute of Crop Science, Rural Development Administration, Muan, Korea in 2015, was used in this study (Lee et al., 2016). Sweetpotatoes (cv. Juwhangmi) obtained from the same center were used in this study. The sweetpotatoes were stored in a moist chamber with 60% relative humidity (RH) at 15°C until use.

To test the *in vitro* inhibitory effect of gaseous ClO_2 against *D. batatas*, 200 μl of spore suspensions was spread on acidified potato dextrose agar (APDA) (three plates per treatment as replicate). For inoculum preparation, spores from cultures of isolate SP-d1 grown on potato dextrose agar (PDA) at 25°C for 14 days were harvested with 0.03% Tween 20 and then adjusted to 5×10^3 spores/ml using a hemocytometer. The inoculated plates were treated with 1, 5, 10, and 20 ppm of ClO_2 gas for 0, 1, 10, 30, and 60 min. The gas was generated by a ClO_2 generator (PurgoFarm Co. Ltd., Hwasung, Korea) (Supplementary Fig. 1) using an electrochemical method (Gates, 1998). Briefly, aqueous NaClO_2 was electrolyzed and the cleaved sodium ions migrated to the cathode through a patented multi-porous membrane electrode assembly, leaving highly pure ClO_2 (> 99%) in the anode chamber. The evaporating ClO_2 gas was blown out through a vent into a test chamber [54 (length) \times 44 (width) \times 46 (height) cm]. Gas entry to the chamber was manually controlled depending on the preset concentrations (1, 5, 10, and 20 ppm) of ClO_2 , which were monitored at 0, 1, 10, 30, and 60 min by a PortaSens II gas leak detector (Analytical Technology, Collegeville, PA, USA) (Supplementary Fig. 1). The ClO_2 gas-treated plates were incubated at 25°C for 2 days and then colony-forming units (cfus) were determined.

To test the *in vivo* inhibitory effect of gaseous ClO_2 against *D. batatas* *via* slice test, healthy sweetpotato roots were first washed with tap water and then with distilled water. The clean roots were surface-sterilized by spraying with 70% ethanol. After drying for 5 min, these roots were rinsed with distilled water twice and then dried for 10 min at room temperature. Next, the roots were cut into 1-cm thick slices and placed in Petri plates (90 mm in diameter). The centers of these slices (three slices per treatment) were drop-inoculated with 10 μl of the spore suspension (5×10^6 spores/ml) harvested as described above. The inoculated slices were then treated with 5, 10, and 20 ppm of ClO_2 gas for 0, 10, 30, and 60 min as described above. After ClO_2 gas treatment, the slices were put in containers [23 (length) \times 13 (width) \times 16 (height) cm] containing three layers of wet paper towels (100% RH) and further incubated at 28°C. After 10 days of incubation, lesion diameters on the inoculated slices were measured as follows: [longest lesion

length (mm) + shortest lesion length (mm)] was divided by 2 (Oh et al., 2016). In addition, fungal populations from the lesions on the slices were assessed. Samples from lesions were obtained using a sterile cork borer and were finely ground using an analytical mill (IKA A11 basic, IKA Works, Wilmington, DE) in sterile distilled water (SDW). After appropriate dilutions, samples were spread on APDA, and cfus were counted 2 days after incubation at 25°C.

To test the *in vivo* inhibitory effect of gaseous ClO₂ against *D. batatas* via the root test, sweetpotato roots were dipped in spore suspensions (5×10^6 spores/ml) or 0.03% Tween 20 (uninoculated control) for 10 min (three roots per treatment). The inoculated roots were treated with 20 and 40 ppm of ClO₂ gas for 0, 30, and 60 min as described above. The concentrations of ClO₂ gas in this root tests were examined to consider practical applications during postharvest storage. After the ClO₂ gas treatment, surface layers of the roots were peeled using a vegetable peeler. Two grams of the samples (approximately 1.5 mm in thickness) were finely ground using an analytical mill (IKA Works) in 20 ml of SDW. After appropriate dilutions, the samples were spread on APDA. After 2 days of incubation at 25°C, colonies were counted and expressed as cfu per g of dry weight.

Experiments were established using factorial designs to observe the effect of ClO₂ concentration and treatment time on lesion diameter and fungal population. All experiments were performed twice with three replicates per treatment. *In vitro* fungal population data from repeated experiments were pooled after confirmation of homogeneity of variances, using Levene's test (Levene, 1960). For analysis of fungal populations, data were analyzed after logarithmic transformation. ANOVA was conducted using general linear model procedures, and the means were separated using least significant difference tests at $P < 0.05$. Statistical analysis of the data was conducted using Statistical Analysis Systems software (SAS Institute, Cary, NC, USA).

The inhibitory effect of gaseous ClO₂ on *D. batatas* SP-d1 was significant (Fig. 1 and Supplementary Fig. 2). Fungal growth was significantly inhibited at 1 ppm of ClO₂ for 1 min and was almost completely suppressed at 1 ppm for 30 min, as well as at other concentrations of ClO₂ treatments, regardless of time (Fig. 1 and Supplementary Fig. 2).

Lesion diameters of *D. batatas* SP-d1 did not change significantly at the concentrations of ClO₂ tested; however, lesion diameters decreased with an increase in treatment time in both experiments (Fig. 2, Fig. 3A, and Table 1). The concentration significantly ($P = 0.0352$) affected lesion diameters in one experiment (Table 1). Treatment time significantly ($P = 0.0046$ for experiment 1 and $P < 0.0001$

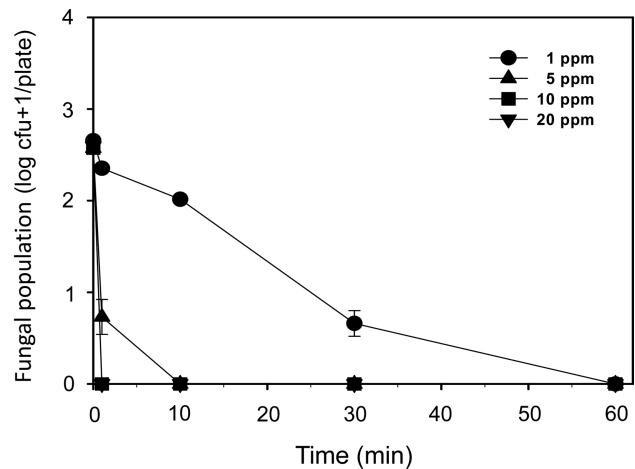


Fig. 1. Populations of *Diaporthe batatas* SP-d1 on acidified potato dextrose agar treated with various ClO₂ concentrations (1, 5, 10, and 20 ppm) for 0, 1, 10, 30, and 60 min. Colony-forming units (cfus) were counted 2 days after incubation. Isolate SP-d1 ($200 \mu\text{l}$ of 5×10^3 spores/ml) was spread on APDA before ClO₂ gas treatments. Error bars are the standard deviations of the means ($n = 6$).

for experiment 2) affected lesion diameters on sweetpotato slices in both experiments. Interactions between concentration and time for lesion diameters were not significant ($P > 0.05$) in both experiments (Table 1). In addition, the degree of darkness of lesions was reduced over time, regardless of gas concentration, and sweetpotato tissues hardened after gas treatment for 60 min (Fig. 2). However, disease symptoms were not observed in uninoculated sweetpotato slices (Fig. 2).

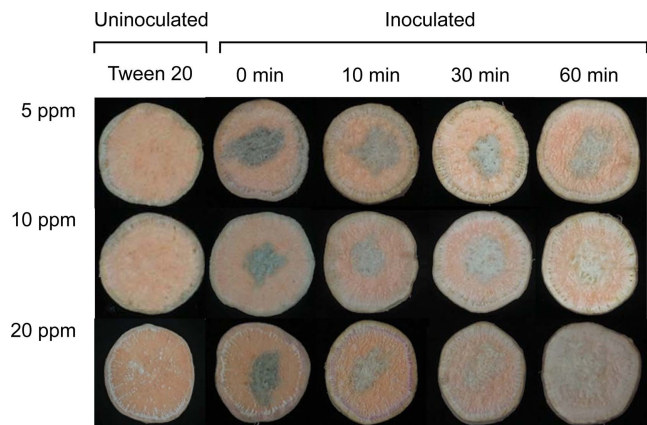
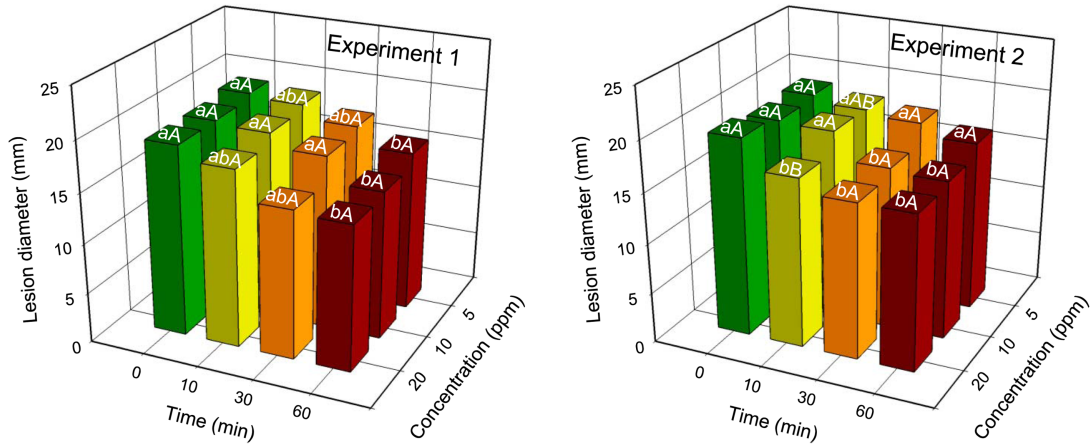
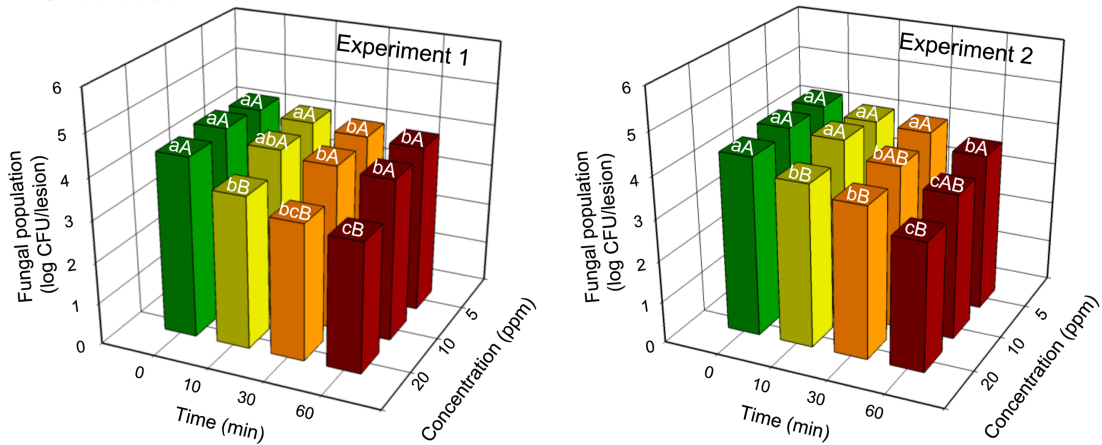


Fig. 2. Photographs of sweetpotato slices drop-inoculated with *Diaporthe batatas* SP-d1 ($10 \mu\text{l}$ of 5×10^6 spores/ml) following treatments with various ClO₂ concentrations (5, 10, and 20 ppm) for 0, 10, 30, and 60 min. These photographs were taken 10 days after inoculation.

A Slice test



B Slice test



C Root test

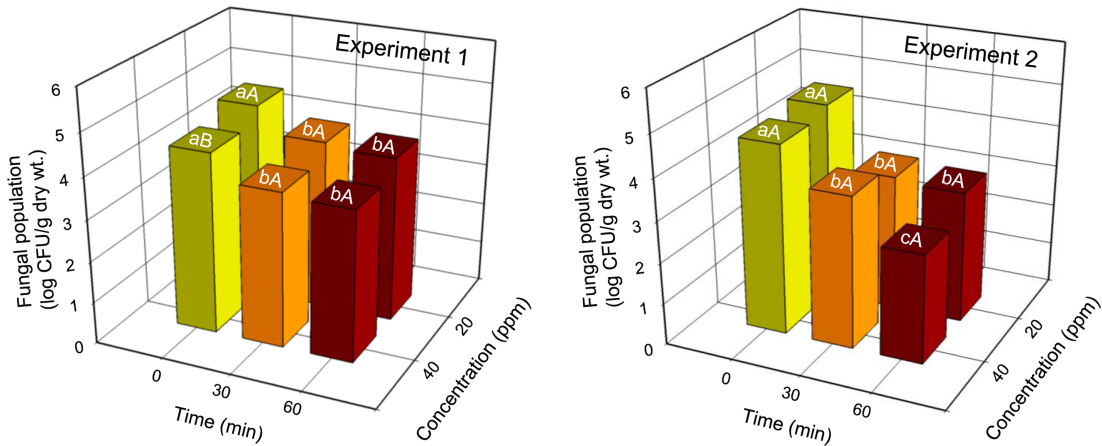


Fig. 3. (A) Lesion diameters and (B) populations of *Diaporthe batatas* SP-d1 on inoculated slices of sweetpotatoes treated with gaseous chlorine dioxide (ClO_2). (C) Populations of isolate SP-d1 in the surface layers of sweetpotato roots treated with ClO_2 gas. Slices were inoculated with isolate SP-d1 ($10 \mu\text{l}$ of 5×10^6 spores/ml) and then treated with various ClO_2 concentrations (5, 10, and 20 ppm) for 0, 10, 30, and 60 min. Roots were dipped in spore suspension (5×10^6 spores/ml) for 10 min and then treated with different ClO_2 concentrations (20 and 40 ppm) for 0, 30, and 60 min. Different lowercase and uppercase letters on bars ($n = 3$) are significantly different between time at a given concentration and between concentrations at a given time according to the least significant difference test at $P < 0.05$, respectively. Repeated experiments are indicated as experiments 1 and 2.

The population of *D. batatas* SP-d1 in the inoculated slices decreased with an increase in ClO₂ concentration and treatment time, regardless of experiment (Fig. 3B and Table 1). Fungal inhibition was higher at 20 ppm of ClO₂ than 5 ppm, regardless of time, and at 30- and 60-min gas treatments compared with 0-min gas treatment, regardless of ClO₂ concentration in both experiments (Fig. 3B). ClO₂ concentrations and treatment times independently resulted in significantly ($P < 0.0001$) reduced fungal growth on sweetpotato slices in both experiments (Table 1). There were significant ($P = 0.0073$) interactions between concentration and time for fungal populations in one experiment (Table 1).

The growth of *D. batatas* SP-d1 did not vary between ClO₂ concentrations; however, it decreased with increasing exposure time in both experiments (Fig. 3C and Table 2). Fungal growth was inhibited at 20 and 40 ppm of ClO₂ with the 60-min gas treatment, compared with the 0-min gas treatment (Fig. 3C). The concentration of ClO₂ resulted in a significant ($P = 0.0012$) reduction in the fungal population in one experiment; the treatment time produced a

significant ($P < 0.0001$) reduction in the fungal population in both experiments (Table 2). However, significant interactions between concentration and time for fungal populations were not observed in both experiments (Table 2).

In this study, we tested whether gaseous ClO₂ could effectively control infection by *D. batatas*, the causal agent of dry rot on sweetpotato. Consequently, we found that gaseous ClO₂ had significant inhibitory activity against the fungal infection on sweetpotato. Furthermore, we detected that treatment time had a greater effect than the concentration of ClO₂ gas on the growth of *D. batatas* SP-d1 and dry rot development in the crop.

In the *in vitro* test, growth of *D. batatas* SP-d1 was partially inhibited at 1 ppm ClO₂ treatment for 30 min. With the 10- and 20-ppm ClO₂ treatments, the tested fungus was completely inhibited. Similarly, there have been several studies that demonstrated the *in vitro* inhibitory efficiency of ClO₂ on fungal pathogens. For example, the radial growth of *Alternaria alternata* and *Stemphylium vesicarium* were completely inhibited after a 3 min treatment with 10 ppm of ClO₂ gas (Trinetta et al., 2013). In other

Table 1. Analysis of variance components including the degrees of freedom (df), sum of squares (SS), *F* ratio, and *P* value for lesion diameters and fungal populations of *Diaporthe batatas* SP-d1 on inoculated slices of sweetpotatoes treated with gaseous chlorine dioxide (ClO₂)

Source of variation	Lesion diameter (mm) ^a								Fungal population (log cfu/lesion) ^a							
	Experiment 1				Experiment 2				Experiment 1				Experiment 2			
	df	SS	<i>F</i>	<i>P</i>	df	SS	<i>F</i>	<i>P</i>	df	SS	<i>F</i>	<i>P</i>	df	SS	<i>F</i>	<i>P</i>
Concentration	2	16.0	1.5	0.2460	2	12.2	3.9	0.0352	2	2.2	32.3	<0.0001	2	0.9	15.6	<0.0001
Time	3	90.9	5.6	0.0046	3	58.0	12.3	<0.0001	3	2.4	23.9	<0.0001	3	3.9	43.5	<0.0001
Concentration × time	6	7.2	0.2	0.9653	6	11.2	1.2	0.3495	6	0.8	3.9	0.0073	6	0.4	2.2	0.0843

^aSweetpotato slices were drop-inoculated with isolate SP-d1 (10 μl of 5 × 10⁶ spores/ml) and then treated with various ClO₂ concentrations (5, 10, and 20 ppm) for 0, 10, 30, and 60 min. Lesion diameters and colony-forming units (cfus) were evaluated 10 days after incubation at 28°C. Repeated experiments are indicated as experiments 1 and 2.

Table 2. Analysis of variance components including the degrees of freedom (df), sum of squares (SS), *F* ratio, and *P* value for fungal populations of *Diaporthe batatas* SP-d1 in surface layers of sweetpotato roots treated with gaseous chlorine dioxide (ClO₂)

Source of variation	Fungal population (log cfu/g dry wt.) ^a							
	Experiment 1				Experiment 2			
	df	SS	<i>F</i>	<i>P</i>	df	SS	<i>F</i>	<i>P</i>
Concentration	1	0.6	17.8	0.0012	1	0.1	0.4	0.5614
Time	2	1.8	29.2	<0.0001	2	9.6	30.3	<0.0001
Concentration × time	2	0.0	0.0	0.9971	2	0.7	2.2	0.1584

^aSweetpotato roots were dipped in spore suspension of isolate SP-d1 (5 × 10⁶ spores/ml) for 10 min and then treated with various ClO₂ concentrations (20 and 40 ppm) for 0, 30, and 60 min. After ClO₂ gas treatment, colony-forming units (cfus) on the surface layers of the roots were determined. Repeated experiments are indicated as experiments 1 and 2.

studies, the impact of atmospheric chlorine on spore viability and mycelial growth of *Botrytis cinerea* and *Rhizopus stolonifer* were evaluated (Avis et al., 2006). A chlorine concentration of 10 ppm was sufficient to inhibit fungal spore germination, while mycelial growth of the fungi was completely inhibited at concentrations of 5 and 20 ppm at 6 h after treatment (Avis et al., 2006).

In the *in vivo* tests, ClO₂ gas consistently restricted lesion diameters for the 60-min treatment, but not for the 10- and 30-min treatments, at concentrations of 10 and 20 ppm in repeated experiments. However, lesion color (indicating fungal growth) on the slices changed over time, and the tissues hardened after gas treatment for 60 min, especially at 20 ppm of ClO₂. Therefore, the fungal populations of infected tissues were evaluated for a more accurate assessment of inhibitory activity of ClO₂ gas. Populations of *D. batatas* SP-d1 greatly decreased at the tested ClO₂ concentrations over time, especially at 20 ppm of ClO₂ for 30-60 min. However, considering tissue hardening after 60 min of ClO₂ treatment, a 30-min treatment may be appropriate. On the other hand, the root dip tests for fungal populations also showed similar results compared with the slice tests as observed in other studies (Jin-Hua et al., 2007; Lee et al., 2004). For example, when green bell peppers were treated with 0-50 ppm of ClO₂ gas at 10 ± 0.5°C for 40 days, pepper rot was inhibited at all of the tested ClO₂ gas concentrations (Jin-Hua et al., 2007). Similarly, reductions in *Escherichia coli*, *Salmonella typhimurium*, and *Listeria monocytogenes* were observed when inoculated lettuce was exposed to ClO₂ gas for 0.5-3 h (Lee et al., 2004).

Taken together, our results in this study showed that gaseous ClO₂ could significantly inhibit *D. batatas* growth and dry rot development on sweetpotato. In particular, 20 ppm of ClO₂ gas treatment for 30 min may be appropriate to inhibit fungal growth and disease development on the crop. Hence, gaseous ClO₂ could be used to control this fungal disease during the postharvest storage of sweetpotato roots.

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