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# Sanitizing radish seeds by simultaneous treatments with gaseous chlorine dioxide, high relative humidity, and mild heat

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ABSTRACT

Sanitizing radish seeds intended for edible sprout production was achieved by applying simultaneous treatments with gaseous chlorine dioxide ($ClO_2$), high relative humidity (RH, 100%), and mild heat (55 °C). Gaseous $ClO_2$ was produced from aqueous $ClO_2$ (0.66 ml) by mixing sulfuric acid (5% w/v) with sodium chlorite (10 mg/mL) in a sealed container (1.8 L). Greater amounts of gaseous $ClO_2$ were measured at 23% RH (144 ppm after 6 h) than at 100% RH (66 ppm after 6 h); however, the lethal activity of gaseous $ClO_2$ against naturally occurring mesophilic aerobic bacteria (MAB) on radish seeds was significantly enhanced at 100% RH. For example, when exposed to gaseous $ClO_2$ at 23% RH, the number of MAB on radish seeds decreased from 3.7 log CFU/g to 2.6 log CFU/g after 6 h. However, when exposed to gaseous $ClO_2$ at 100% RH for 6 h, the MAB population decreased to 0.7 log CFU/g after 6 h. Gaseous $ClO_2$ was produced in higher amounts at 55 °C than at 25 °C, but decreased more rapidly over time at 55 °C than at 25 °C. The lethal activity of gaseous $ClO_2$ against MAB on radish seeds was greater at 55 °C than at 25 °C. When radish seeds were treated with gaseous $ClO_2$ (peak concentration: 195 ppm) at 100% RH and 55 °C, MAB were reduced to populations below the detectable level (<— 0.7 log CFU/g) within 2 h without decreasing the seed germination rate (97.7%). The lethality of combined treatments against artificially inoculated *Escherichia coli* O157:H7 was also evaluated. When exposed to gaseous $ClO_2$ at 100% RH and 55 °C for 6 h, the initial number of *E. coli* O157:H7 (3.5 log CFU/g) on radish seeds decreased to below the detection limit (0.7 log CFU/g) by direct plating but it was not eliminated from seeds. The germination rate of radish seeds was not significantly ($P > 0.05$) decreased after treatment for 6 h. The information reported here will be useful when developing decontamination strategies for producing microbiologically safe radish seed sprouts.



## 1. Introduction

Seed sprouts have become popular among health-conscious consumers in recent years because they are easy to produce and contain substantial quantities of vitamins, minerals, and proteins (Kim et al., 2009b; Neetoo and Chen, 2010). With increased consumption of alfalfa, mung bean, radish, fenugreek, and other types of seed sprouts, there has been a concurrent increase in the number of sprout-associated outbreaks of diseases (Dechet et al., 2014; Taormina et al., 1999). The largest sprout-associated outbreak occurred in Sakai, Japan in 1996. Radish sprouts contaminated with *Escherichia coli* O157:H7 were the causative vehicle, which resulted in 9,451 cases and 12 deaths (Michino et al., 1999). More recently, a fenugreek sprout-associated outbreak of shiga toxin-producing *E. coli* O104:H4 infections in Germany resulted in 3,842 cases and 53 deaths (Muniesa et al., 2012).

There are several possible routes of transmission of foodborne pathogens into seed sprouts. Foodborne pathogens may be present on the seeds before sprouting, contaminate sprouts during production, or be introduced during handling of sprouts (Kim et al., 2009a). Among these routes, it has been suspected that the major source of foodborne pathogens in most sprout-associated outbreaks is contaminated seeds (National Advisory Committee on Microbiological Criteria for Foods, 1999; U.S. Food and Drug Administration, 2014). Seeds can be contaminated with various pathogenic microorganisms during cultivation, harvesting, scarification, transport, and distribution. Animal excrement, runoff from animal production facilities, untreated agricultural water, improperly composted manure, dirty equipment, and the environment can be sources of foodborne microbial contaminants (Bari et al., 2011). The National Advisory Committee on Microbiological Criteria for Foods recommends the incorporation of a treatment, e.g., soaking seeds in

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calcium hypochlorite (20,000 ppm), capable of causing a 5-log reduction in levels of *Salmonella* and enterohemorrhagic *E. coli* O157 (National Advisory Committee on Microbiological Criteria for Foods, 1999). Presoaking seeds in calcium hypochlorite results in a marked decrease in the number of pathogenic microorganisms, but does not assure elimination (Brooks et al., 2001; Weissinger and Beuchat, 2000). Even if the initial number of a foodborne pathogen is extremely low (e.g., <−1 log CFU/g), the warm temperature, high humidity, and high nutrient availability during sprout production are favorable for growth (Choi et al., 2016). The ultimate solution for preventing sprout-associated outbreaks is to eliminate foodborne pathogens on seeds and to prevent contamination during sprouting and subsequent handling.

Chlorine dioxide ($ClO_2$) is a strong oxidant that has been used to successfully sanitize fruits and vegetables (Gómez-López, 2012). Both gaseous and aqueous forms of $ClO_2$ are commercially available. Compared to aqueous $ClO_2$, gaseous $ClO_2$ has some advantages. For example, gaseous $ClO_2$ is superior to aqueous $ClO_2$ in reaching microorganisms in concealed by surface irregularities or protected in biofilms (Gómez-López, 2012). Han et al. (2001b) demonstrated that, due to its greater ability to penetrate, gaseous $ClO_2$ is more effective than is aqueous $ClO_2$. The efficacy of gaseous $ClO_2$ against *Salmonella enterica* and *E. coli* O157:H7 inoculated on tomato, cantaloupe, and lettuce seeds has been reported (Trinetta et al., 2010, 2011). Other studies have shown that treatment with aqueous $ClO_2$ in combination with other stress conditions such as drying, wet heat or dry heat has a synergistic lethal effect against foodborne pathogens on radish seeds (Bang et al., 2011a, 2011b, 2011c; Kim et al., 2010). However, the lethal effects of gaseous $ClO_2$ in combination with other stress treatments on microorganisms present on seeds intended for sprout production have not been fully described.

The aim of this study was to evaluate simultaneous treatments of gaseous $ClO_2$, high RH, and mild heat in decreasing the numbers of indigenous mesophilic aerobic microorganisms (MAB) and artificially inoculated *E. coli* O157:H7 on radish seeds (<−0.7 log CFU/g) without decreasing their germination rate. To achieve this goal, we investigated: i) the influence of RH (23 and 100%) on production of gaseous $ClO_2$ from aqueous $ClO_2$ and its lethality against MAB on radish seeds, ii) the influence of temperature (25 and 55 °C) on the production of gaseous $ClO_2$ and its lethality against MAB on radish seeds, and iii) the lethality of simultaneous treatment with gaseous $ClO_2$, high RH (100%), and high temperature (55 °C) against MAB and *E. coli* O157:H7 on radish seeds and its influence on the germination rate of the seeds.

## 2. Materials and methods

### 2.1. Preparation of E. coli O157:H7 inoculum

Five nalidixic acid-adapted strains of *E. coli* O157:H7 were used: ATCC 43895 (from hamburger), E0018 (from cattle faeces), F4546 (from a patient in an alfalfa sprout associated outbreak), H1730 (from a lettuce-associated outbreak), and 932 (from a patient with haemorrhagic colitis). Cryopreserved *E. coli* O157:H7 strains were activated in 10 ml of tryptic soy broth (TSB; BBL/Difco, Sparks, MD, USA) at 37 °C for 24 h. Each of the five activated strains was transferred to 10 ml of TSB containing nalidixic acid (50μg/ml, TSBN) at 37 °C at three consecutive 24-h intervals. An *E. coli* O157:H7 cocktail (10 ml) was prepared by combining 2 ml of each of the five strains. The cocktail was centrifuged at 2000 × *g* for 15 min at room temperature (22 ± 2 °C), supernatants were decanted, and cells were resuspended in 10 ml of sterile distilled water (DW). This procedure was repeated, and the suspensions were serially diluted in DW to prepare *E. coli* O157:H7 inoculum (ca. 7 log CFU/ml).

### 2.2. Construction of treatment containers

Sealed containers (1.8 L; 142 mm diameter by 186 mm high; Lock & Lock Co. Ltd., Seoul, Republic of Korea) in which radish seeds were treated were constructed as described by Nam et al. (2014). In brief, the containers were comprised of three components: one from which gaseous $ClO_2$ was removed and analyzed for concentration, a second in which gaseous $ClO_2$ (23 or 100% RH) is produced, and a third in which radish seeds are treated (Fig. 1). A Petri dish containing aqueous $ClO_2$ served as the $ClO_2$ production component; gaseous $ClO_2$ was produced by spontaneous vaporization. Above this component, radish seeds were placed in a sterile lid with a wire-screened ventilation hole through which gaseous $ClO_2$ passed (Fig. 1).

### 2.3. Preparation of relative humidity (RH) environments

To investigate the influence of RH on the production of gaseous $ClO_2$ and its lethality to MAB on radish seeds, RHs in the containers were adjusted to 23, 43, and 100% as described by Kim et al. (2008). Briefly, saturated potassium acetate ($a_w$ 0.23, Daejung, Siheung, Republic of Korea) solution, saturated potassium carbonate ($a_w$ 0.43, Daejung) solution, and sterile DW were prepared to give $a_w$ values of 0.23, 0.43, and 1.00, respectively, with corresponding atmospheric equilibrium RHs of 23, 43, and 100%. Salt solutions or DW (150 ml) were deposited in the containers followed by incubating at 25 °C or 55 °C for at least 24 h before initiation of experiments.

### 2.4. Influence of RH on the production of gaseous $ClO_2$ and its lethality against MAB on radish seeds

#### 2.4.1. Influence of RH on the production of gaseous $ClO_2$

Gaseous $ClO_2$ was generated from aqueous $ClO_2$ by spontaneous vaporization. Aqueous $ClO_2$ was prepared by combining 8 ml of 5% sulfuric acid (95% assay, Wako Pure Chemical Industries, Ltd., Osaka, Japan) with 0.1 g of sodium chlorite (Technical grade 80%, Sigma-Aldrich, St. Louis, MO, USA) and vortexing at maximum speed for 1 min. The mixture (0.66 ml) was deposited in a Petri dish in containers at 23 or 100% RH (Fig. 1). Containers were tightly sealed and held at 25 °C for up to 6 h. After 1, 2, and 6 h, concentrations of gaseous $ClO_2$ in treatment containers were measured using a gas detector pump and tube (model 8 H, Gastec Corporation, Tokyo, Japan) as instructed by the manufacturer. The gas detector pump was used to collect the internal air from the container and a gas detector tube was used to measure the concentration of gaseous $ClO_2$ in the collected air.

#### 2.4.2. Influence of RH on the lethality of gaseous $ClO_2$ against MAB on radish seeds

Radish seeds were purchased online (Saessakmart, Seoul, Republic of Korea; www.saessakmart.co.kr). Five grams of seeds were placed on sterile lids fitted with wire-screened ventilation holes in containers controlled at 23 or 100% RH and incubated at 25 °C for 24 h. After 24 h, 0.66 ml of aqueous $ClO_2$ was placed in a Petri dish below the lid containing radish seeds and the containers were sealed (Fig. 1). The containers with radish seeds exposed to gaseous $ClO_2$ were held at 25 °C for 1, 2, and 6 h. At each sampling time, seeds (5 g) were transferred to a Whirl-pak® bag (207 ml; Nasco, Fort Atkinson, WI, USA) containing 25 ml of TSB and pummeled for 1 min. Undiluted suspensions (0.25 ml in quadruplicate and 0.1 ml in duplicate) and suspensions (0.1 ml in duplicate) serially diluted in 0.1% peptone water (PW) were surface-plated on tryptic soy agar (TSA; BBL/Difco) and incubated at 37 °C for 24 h before counting colonies. The remaining mixtures of TSB and seeds were incubated at 37 °C for 24 h to enrich the MAB. If unenriched mixtures did not form colonies on TSA, enriched mixtures were streaked onto TSA and incubated at 37 °C for 24 h. The theoretical detection limits for MAB using direct plating and enrichment were 5 CFU/g (0.7 log CFU/g) and 1 CFU/5 g (−0.7 log CFU/g), respectively.

To determine the effects of gaseous $ClO_2$ and RH on germination rate, radish seeds (n = 100) exposed to gaseous $ClO_2$ at 23 or 100% RH for 1, 2, or 6 h were placed onto a sterile cheese cloth in a