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Efficacy of Aqueous Chlorine Dioxide and Citric Acid in Reducing *Escherichia coli* on the Radish Seeds Used for Sprout Production

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Abstract The efficacy of citric acid-aqueous chlorine dioxide (ClO_2) treatment of radish seeds artificially contaminated with *Escherichia coli* was studied. Radish seeds were inoculated with *E. coli*. Following inoculation, samples were stored at 4°C and soaked in citric acid or aqueous ClO_2 for 10 min. The treatment of radish seeds using 200 ppm aqueous ClO_2 solution caused a 1.5 log CFU/g reduction in the population of *E. coli*. Compared to the aqueous ClO_2 treatment, soaking radish seeds in 2.0% citric acid solution for 10 min was more effective in reducing *E. coli* populations on radish seeds. The efficacy of spray application of chlorine (100 ppm) or 0.5% citric acid to eliminate *E. coli* during the germination and growth of radish was investigated. Radish seed inoculated with *E. coli* was treated for the duration of the growth period. Although it resulted in a decrease in the *E. coli* population, the spray application of 100 ppm chlorine during the growth period was not significantly effective. In contrast, the combined treatment of seeds using 200 ppm aqueous ClO_2 and treatment of sprouts with 0.5% citric acid solution during sprout growth was hardly effective in eliminating *E. coli*.

Keywords: aqueous chlorine dioxide (ClO_2), citric acid, sprout, radish, seed, *Escherichia coli*

Introduction

In recent years, there has been an increasing concern regarding the safety of foods especially products that are consumed fresh or slightly cooked. According to the Center for Disease Control and Prevention, approximately 76 million cases of food-borne diseases in the US every year result in 5,000 deaths (1). Moreover, there has been an increase in the frequency of outbreaks of *Salmonella* and *Escherichia coli* O157:H7 illness associated with the consumption of raw seed sprouts (2). The largest outbreak reported to date involved approximately 6,000 people and resulted from the consumption of contaminated radish sprouts (3).

The chemical treatment of seeds to kill pathogenic bacteria that may be present in the seeds has been investigated by a number of researchers (4-7). The use of rinse water chlorinated at 100 ppm during the sprouting of mungbean decreased the counts of natural microflora by <1 log (8). Food and Drug Administration (FDA) (9) allows the use of aqueous chlorine dioxide (ClO_2) in washing fruits and vegetables. At a concentration >100 ppm, acidified ClO_2 significantly reduced the populations of *E. coli* O157:H7 on seeds. A 500 ppm ClO_2 reduced the pathogen from 2.7 to <0.5 log CFU/g (10).

The conditions under which sprouts are grown are conducive for the growth of many types of bacteria such as *E. coli, Salmonella,* and other enteric pathogens. Thus, if seed disinfection does not eliminate the target pathogen completely, then the organism may grow during germination and sprout growth to levels that may cause human illness (11). Andrew *et al.* (12) and Jaquette *et al.* (6) reported an approximately 3-5 log increase in *Salmonella* during the growth period when the seeds were inoculated with the pathogen. Likewise, studies on pathogens suggest increased unacceptable levels in mature sprouts. Pathogens proliferating during germination and sprouting may be practically impossible to eliminate on the mature sprout. Research wherein sprouts were inoculated with *E. coli* O157:H7 and *Salmonella* found pathogen in the inner tissue of experimentally contaminated sprouts during their growth (11,13). The treatment of the outer surface of sprouts using mercury chloride did not kill the *E. coli* O157:H7 located on the subsurface locations (14). Gandhi *et al.* (11) reported that the treatment of seeds using 20,000 ppm chlorine >5 log reduced the population of *Salmonella* but did not eliminate the pathogen.

Organic acids also have an antimicrobial activity, since their ability to lower the pH result in the instability of bacterial cell membranes (14-16). The widely acknowledged importance of eliminating microbial counts has led to the use of lactic acid as a decontaminating agent in the food industry (17). Treatment using acetic acid effectively reduced *E. coli* O157:H7 and *Salmonella typhimurium* on the carcass surfaces (18-20). Prasai *et al.* (20) reported that the pronounced antibacterial effects of lactic acid on limiting the growth of bacteria were enhanced at colder storage temperatures.

Alternative methods of controlling pathogenic bacteria on seed sprouts are required. Using treated seeds as well as a regime that would either prevent the growth of or kill the target pathogen during germination and sprouting would increase the microbiological safety of seed sprouts.

This study sought to determine the efficacy of aqueous ClO_2 in killing *E. coli* inoculated on radish seeds. It also aimed at investigating and comparing the effects of 100 ppm chlorine solution and combined organic acid treatment followed by aqueous ClO_2 during germination and growth on reducing or eliminating *E. coli* on radish sprouts.

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Materials and Methods

Bacterial strain and preparation of inoculum *Escherichia coli* ATCC 9637 was used for the analysis. The cell was grown in a brain heart infusion broth (Difco, Becton, Dickinson Co., Sparks, MD, USA) at 37°C for 24 hr. The *E. coli* stain was prepared from 1 L of the result of 24 hr culturing and centrifuging (Sorvall RC28S; Dupont Co., Wilmington, DW, USA) at 4°C and 3,300×g for 15 min. The supernatant was decanted, and the pellet, resuspended in 300 mL of sterile 0.1% peptone water before centrifuging again at 3,300×g for 15 min at 4°C. The pellet was then resuspended in 1 L of sterile 0.1% peptone water.

Inoculation of radish seeds Radish seeds (1 kg) (Asia Seed Co., Seoul, Korea) were combined with a bacterial cell suspension (1 L) and gently mixed for 10 min on a rotator platform. The suspension was then decanted, and seeds were placed on a wire mesh to dry at 21-23°C under a laminar flow hood for 24 hr. Ten g of dried seeds were pummeled in 90 mL of 0.85% NaCl water for 2 min and surface-plated on Chromocult agar (ChromocultR; Merck Co., Darmstadt, Germany). The plates were incubated for 24 hr at 37°C. An *E. coli* population of ca. 10^7 CFU/g seeds was obtained. Dried seeds were sealed in plastic Ziploc bags, stored at 4°C, and used within 2 weeks.

Preparation of aqueous ClO_2 water Aqueous ClO_2 water was produced with an aqueous ClO_2 generator (Bupyuk & Tech. Co., Seoul, Korea) using 2% sodium chlorite and ClO_2 gas. The concentration of aqueous ClO_2 was measured using an iodometry standard method (21).

Treatment of radish seeds and sprouts Twenty g of *E. coli*-inoculated radish seeds were treated with 180 mL aqueous ClO_2 water through continuous agitation using a motorized stirring rod at a speed setting of 3 (PC-410; Corning Co., Nagog Park Acton, MA, USA). Various levels of aqueous ClO_2 concentration were used to study the effect of aqueous ClO_2 characteristics on the pathogens present on radish seeds.

Efficacy of washing treatments during sprouting Inoculated seeds were spread on screen trays and placed in a fabricated germination and sprout growth chamber. The temperature of the chamber was maintained at 25°C. Seeds were spray-irrigated with distilled water, water containing 100 ppm chlorine, or 0.5% citric acid water.

Microbiological analysis Treated radish seeds (10 g) were rinsed with 100 mL sterile deionized water. The rinsed seeds were then combined with 90 mL of 0.85% NaCl solution in a stomacher bag and pummeled for 2 min using a stomacher (400 circulator; Seward Co., Worthing, WS, UK). One mL of stomached seeds slurry was serially diluted in 9 mL sterilized 0.85% NaCl solution and spread-plated on Chromocult agar (Chromocult®; Merck). The plates were incubated at 37°C for 24 hr.

Statistical analysis Experimental data were subjected to the analysis of variance (ANOVA) using the SAS program (SAS) (22). When ANOVA revealed a significant effect at $p<0.05$, the data were further analyzed using Duncan's multiple comparison test.

Results and Discussion

Population of *E. coli* recovered from inoculated radish seeds after treatment Table 1 lists the changes in the *E. coli* populations following treatment using aqueous ClO_2. The method of inoculation used in this study resulted in an initial *E. coli* count of approximately 7 log CFU/g. The reduction in the population of *E. coli* subjected to control (deionized water) treatment ranged from 0.48 to 0.59 log CFU/g (Table 1).

In this study, aqueous ClO_2 (200 ppm for 10 min) was significantly ($p<0.05$) effective in reducing (1.61 log CFU/g reductions) the populations of *E. coli* on seeds. Increasing the washing time (2-40 min) of seeds with 50 and 100 ppm aqueous ClO_2 had no effect on population reduction, however. Still, the treatment using 200 ppm aqueous ClO_2 resulted in population reductions ranging from 0.85 to 2.43 log CFU/g for up to 40 min. In terms of the treatment time, reductions in population for 2 and 5 min treatments were significantly higher ($p<0.05$) than those for 10-40 min. Longer contact caused more *E. coli* to be released from the seeds; hence the higher initial counts compared to shorter contact times. Further increasing the concentration of aqueous ClO_2 (500 ppm for 10 min) also led to a significant ($p<0.05$) decrease (2.73 log CFU/g reduction) in microbial populations. Similar results were reported by Taormina and Beuchat (10), who observed that the 3 min treatment using 500 ppm acidified ClO_2 reduced the population of *E. coli* on alfalfa seeds by more than 2

Table 1. Reduction in counts of *E. coli* on radish seeds treated with various aqueous ClO_2

Treatment time (min)	*E. coli* counts (log CFU/g) after treatment[1)]					
	Distilled water	Concentrations of aqueous ClO_2				
		50 ppm	100 ppm	200 ppm	300 ppm	500 ppm
0	a7.22±0.08A	ab7.02±0.16A	ab7.24±0.21A	ab6.99±0.15A	b6.68±0.17A	b6.65±0.41A
2	a6.63±0.22B	b6.16±0.23C	a6.75±0.42B	b6.14±0.14B	a6.68±0.05A	b6.06±0.12B
5	a6.42±0.06B	ab6.40±0.15C	a6.56±0.29B	b6.15±0.02B	ab6.29±0.08AB	c5.00±0.03C
10	ab6.33±0.06B	b6.24±0.08C	a6.71±0.06B	c5.38±0.18C	c5.91±0.60B	d3.92±0.21D
20	a6.31±0.18B	a6.39±0.21C	a6.50±0.13B	b5.57±0.31C	c4.86±0.27C	d3.60±0.27D
40	a6.43±0.30B	a6.78±0.04C	a6.30±0.13B	b4.56±0.51D	b4.77±0.85C	c1.10±0.17E

[1)]Different letters (A-E) and (a-d) are significantly different ($p<0.05$) within the same column and row, respectively.

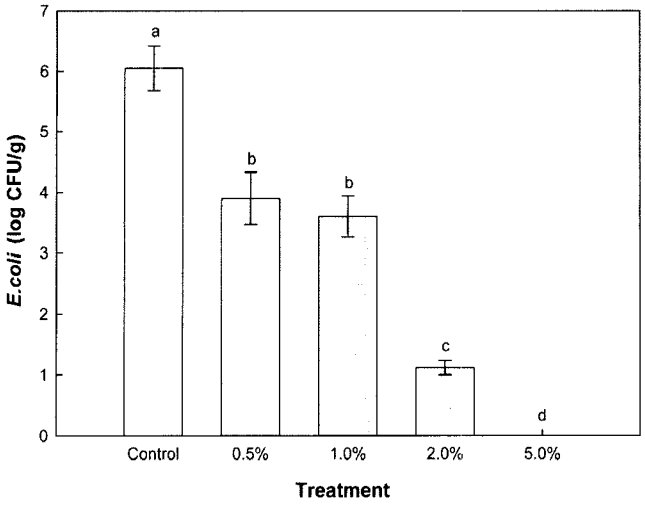

Fig. 1. Reduction of *E. coli* recovered from inoculated radish seeds after treatment with citric acid of various concentrations for 10 min. Values with the different letters are significantly different ($p<0.05$).

log CFU/g.

Figure 1 shows the changes in *E. coli* populations due to treatment with citric acid. The treatments of seeds using the recommended 10 min exposure at different concentrations resulted in population reductions ranging from 2.35 to 6.05 log CFU/g. *E. coli* populations on inoculated seeds were 6.05 log CFU/g; they decreased to 1.12 log CFU/g judging from the agar plate count on seeds treated with 2.0% citric acid. Although the addition of 5.0% citric acid to *E. coli*-contaminated radish seeds completely inhibited the growth of the organism, the condition of high concentration clearly reduced the sprouting rate (under 50%). However, the sprouting rate of the addition of 2.0% citric acid to *E. coli*-contaminated radish seed is at least 85%. It means the effect about the sprouting rate at 2.0% citric acid may be not big. Therefore, the condition of high concentration of citric acid may be not the condition which is suitable.

The 2.0% citric acid treatment was more effective than the 500 ppm aqueous ClO_2 treatment. The inability of washing treatments to kill *E. coli* O157:H7 is attributed to the nature of seeds where bacteria can survive (10,23). Furthermore, the treatment of seeds using 20,000 ppm $Ca(OCl)_2$ reportedly results in a significant decrease in the number of the target pathogen but does not completely eliminate the organism (4,7,24,25).

Efficacy of using different sanitizers as irrigation water on radish seeds during sprouting to eliminate *E. coli*

Compared to radish seeds treated using sterile deionized water, other treatments significantly ($p<0.05$) reduced the inoculated population after washing (Table 2). Radish seeds inoculated with *E. coli* were treated with selected sanitizers, i.e., 2.0% citric acid, aqueous ClO_2 (200 ppm for 10 min). The washing of radish sprouts using sterile deionized water as irrigation water for 10 min did not result in a significant change in microbial populations after 48 and 96 hr of sprouting. The population of *E. coli* was estimated to be 7.43 log CFU/g after a 48 hr sprouting period of artificially contaminated seed. The spray application of a 100 ppm solution of chlorine on a sprouted seed exhibited limited efficacy, resulting in less than a 0.1 log reduction in the *E. coli* population. Moreover, there was no significant difference in the populations recovered after sprouting (96 hr) compared to sterile deionized water. *E. coli* cells proliferated (7.95 log CFU/g) during the sprouting process. Similar results were reported by Lang *et al.* (24), who observed that *E. coli* O157:H7 counts increased to 10^7-10^8 CFU/g during sprouting. Itoh *et al.* (13) demonstrated that *E. coli* O157:H7 spread throughout the tissue when radish sprouts were grown from seeds contaminated by such bacteria; they were present not only on the outer surfaces but also inside the cotyledons and stomata. Due to such wide distribution of *E. coli* in radish sprouts, surface sanitizing agents can hardly reach the target pathogen at subsurface locations. The treatment of seeds to eliminate pathogens prior to germination and sprouting has proven to be unsuccessful (4,6,7,26). Similarly, the treatment of mature sprouts to eliminate pathogenic bacteria results in a limited decrease in the population of the target pathogen (11).

Alternatives to or modifications of existing strategies for sanitizing seeds prior to germination and sprouting or sanitization of mature sprouts are essential in ensuring the microbial safety of seed sprouts. The initial populations of *E. coli* on radish seeds were 6.45 and 8.39 log CFU/g. Since pathogens can survive the surface sanitization treatment of seeds and multiply to hazardous levels during sprout production (5), the treatment of sprouts during their growth may prove to be beneficial. Several studies demonstrate that *E. coli* O157:H7 grow extremely well in the sprout environment (5,11).

In this study, the sanitization of sprouts during their growth was investigated. Table 3 shows the results for the populations of *E. coli* recovered from radish sprouts sanitized on various days of maturity. The populations of

Table 2. Detection of *E. coli* in association with radish sprouts grown from seeds with different sanitizer for 10 min

Treatment[1)]	Population on seeds		Population on sprouts	
	After inoculation	After treatment	Day 2	Day 4
Control A	6.37	6.71	7.43	7.86
Control B	6.37	6.71	7.68	7.79
Aqueous ClO_2 200 ppm	8.40	6.61	7.52	7.95
Citric acid 2%	8.40	4.56	7.53	7.84

[1)]Seeds were treated with different sanitizer and treated with distilled water during sprout growth; control B, seeds were treated with distilled water and treated with 100 ppm chlorine during sprout growth; values expressed as log CFU/g.

Table 3. Effect of aqueous ClO_2 and citric acid treatment during germination and sprout growth on *E. coli* populations

Treatment[1)]	Population on seeds		Population	
	After inoculation	After treatment	Germination	Sprouts[2)]
Control A[3)]	6.45[4)]	6.73	7.14	7.30
Aqueous ClO_2 200 ppm	8.39	6.63	6.65	6.67
Citric acid 2%	8.39	4.70	6.88	7.12

[1)]Seeds were treated with different sanitizers and treated with 0.5% citric acid during germination and sprout growth.
[2)]Sprouts were grown for 4 days before microbiological analysis.
[3)]Seeds were treated with distilled water and treated with 100 ppm chlorine during germination and sprout growth.
[4)]Values expressed as log CFU/g.

E. coli recovered from inoculated seeds subjected to selected washing treatments using aqueous 200 ppm ClO_2 and 2.0% citric acid followed by treatment with 0.5% citric acid during sprouting were significantly lower than the initial population (Table 3). On the other hand, seeds treated with sterile deionized water did not contain significantly lower populations compared to unwashed samples.

When seeds were germinated and sprouts were harvested after 96 hr, the number of *E. coli* increased to >7 log. Although values reflecting changes in the population of *E. coli* were not provided, a similar study demonstrated that the germination of treated seeds resulted in contaminated sprouts (Table 3). In practical terms, treatment during the growth of sprouts was only marginally effective in reducing the population of *E. coli* associated with growing sprouts as what the results of this study suggest. The populations of *E. coli* on radish seeds after a 96 hr sprouting period were significantly higher compared to those recovered after treatment. Nonetheless, washing treatments for sprouted seeds using different sanitizers significantly reduced the populations of *E. coli* after 48 hr of germination. It will be able to guess for one of reasons of this phenomenon that acid adaptation response caused an effect. Acid adaptation response is a phenomenon by which microorganisms show an increased resistance to environmental stress after the exposure to a moderate acid environment. Acid adaptation and increased resistance to acid stress have been observed in various organisms including *Listeria* (27-29), *E. coli* (30,31), and *Salmonella* (32). It means the following research about this phenomenon will be necessary.

Castro-Rosas and Escartin (33) reported that the daily irrigation of radish sprouts with 100 ppm ClO_2 did not reduce the populations of mesophilic aerobes over an 8 day growing period. Note, however, that washing with 200 ppm aqueous ClO_2 followed by treatment with 0.5% citric acid significantly reduced the *E. coli* populations during 48 hr of germination. The populations of *E. coli* on radish seeds at the last sample time (96 hr) were also significantly lower (1.7 log CFU/g) compared to the initial population.

In conclusion, the elimination of *E. coli* from a laboratory-inoculated radish seed seems to be problematic. Treatments of radish seeds with different sanitizers before and during the sprouting of seed did not result in the complete eradication of *E. coli* from the radish seed. Nonetheless, the aqueous ClO_2 treatment followed by citric acid treatment tested in this study before and during the sprouting of radish seeds was found to be significantly more effective in the removal of *E. coli*. Thus, further study can help define more effective ways of decontaminating radish seeds and sprouts.

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