

Using Aqueous Chlorine Dioxide To Prevent Contamination of Tomatoes with *Salmonella enterica* and *Erwinia carotovora* during Fruit Washing[†]

S. PAO,* D. F. KELSEY, M. F. KHALID, AND M. R. ETTINGER

Virginia State University, Agricultural Research Station, P.O. Box 9061, Petersburg, Virginia 23806, USA

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ABSTRACT

Chlorine dioxide (ClO₂) is an antimicrobial agent recognized for its disinfectant properties. In this study, the sanitizing effects of ClO₂ solutions against *Salmonella enterica* and *Erwinia carotovora* in water, on tomato surfaces, and between loads of tomatoes were evaluated. In water, ClO₂ at 5, 10, and 20 ppm caused a ≥5-log reduction of *S. enterica* within 6, 4, and 2 s, respectively. Higher lethality was observed with *E. carotovora*; a 5-log reduction was achieved after only 2 s with 10 ppm ClO₂. On fruit surfaces, however, the sanitizing effects were compromised. A full minute of contact with ClO₂ at 20 and 10 ppm was required to achieve a 5-log reduction in *S. enterica* and *E. carotovora* counts, respectively, on freshly spot-inoculated tomatoes. On inoculated fruit surfaces, populations decreased >3 log CFU/cm² during desiccation at 24 ± 1°C for 24 h. Populations of air-dried *Salmonella* and *Erwinia* were not significantly reduced (*P* > 0.05) by ClO₂ at ≤20 ppm after 1 min. Either wet or dry inoculum of these two pathogens could contaminate immersion water, which in turn can cross-contaminate a subsequent load of clean fruit and water. ClO₂ at 5 ppm used for immersion effectively prevented cross-contamination. Pathogen contamination during fruit handling is best prevented with an effective disinfectant. Once a load of fruit is contaminated with pathogens, even a proven disinfectant such as ClO₂ cannot completely eliminate such contaminants, particularly when they are in a dehydrated state on fruit.

Fruits are susceptible to natural contamination from soil, insects, birds, water, and other sources during growth and harvest (6, 20, 24, 30). Although most environmental microbes found on raw fruit are considered benign, contamination with human or plant pathogens presents a persistent challenge to the fresh fruit industry. Inadequate fruit packing, processing, and sanitation can result in further spread of pathogens, leading to serious cross-contamination and outbreaks (13, 23).

Contaminated raw tomatoes have caused several large outbreaks of human salmonellosis over the past few years. In 1999, raw restaurant-prepared tomatoes were implicated in a multistate outbreak of *Salmonella enterica* infection (11). In summer 2004, three outbreaks of *Salmonella* infection associated with eating Roma tomatoes were detected in the United States and Canada (10). In investigations of such widespread outbreaks, researchers often conclude that contamination likely occurred on the farm or during packing, where more effective disinfection and prevention strategies are needed.

Erwinia carotovora is a causative agent of postharvest soft rot decay in many types of produce, including tomatoes (2, 7). Contamination can occur during harvest operations where harvest-associated wounds can introduce the organism to otherwise sound fruit. Risk of cross-contamination

and infiltration of this bacterium into tomatoes is a major concern during packinghouse dumping and hydrocooling operations (4, 13, 29). Chlorine compounds can be used to help control tomato decay caused by *E. carotovora* (5). Standard recommendations call for tomato dump tanks to be maintained at a temperature at least 10°F (5.6°C) above that of incoming fruit to reduce the potential for dump tank solution infiltration (5, 29, 31). The microbial quality of the water is typically maintained by use of sanitizing chemicals registered with the U.S. Environmental Protection Agency. The sanitizer concentration must be closely monitored to ensure adequate levels are maintained to prevent a buildup of microorganisms in the recycled solution that could contaminate fruit.

The primary biocide used in fresh produce packing operations is chlorine, typically in the form of sodium hypochlorite (bleach). Chlorine dissociates in water to either hypochlorous acid or hypochlorite ion, depending upon solution pH (5, 27). The biocidal form of chlorine is hypochlorous acid, and the pH of chlorine solutions must be properly controlled to ensure that adequate levels of the active biocide are maintained. One disadvantage of chlorine is that it can react with trace amounts of organic material on fresh produce to form potentially carcinogenic organochlorine compounds (27, 30). Therefore, alternative sanitizers are being adapted by the produce industry as chlorine replacements.

Chlorine dioxide (ClO₂) is a biocide with 2.5 times the oxidation capacity of chlorine (27). It has been used since

* Author for correspondence. Tel: 804-524-6715; Fax: 804-524-5186; E-mail: spao@vsu.edu.

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the 1950s in drinking water purification and is permitted in wash water for produce (8). In contrast to chlorine, ClO_2 is effective across a broad pH range and does not react with water, remaining a dissolved gas in solution (1). Furthermore, ClO_2 does not react with ammonia to form chloramines nor does it form trihalomethanes in the presence of organic matter.

Chlorine dioxide must be generated on site; thus, past use has been largely restricted to municipal drinking water and pulp and paper industries, where large capacities are required and the significant capital investment for generation systems can be absorbed. However, recently developed methods for generating ClO_2 have made the use of this compound easier and more affordable for the produce industry (19, 26). Some of the methods deliver ClO_2 in a mixture with acids or other compounds; these mixtures are not considered pure ClO_2 sanitizer.

Other researchers have reported antimicrobial effects of aqueous ClO_2 on *Escherichia coli* O157:H7 and *Listeria monocytogenes* on apples, lettuce, strawberries, and cantaloupe (27), *E. coli* on oranges (22), total microorganisms on cucumbers (25), and fungal contaminants on hard surfaces such as belts and pads in a commercial apple and pear packinghouse (26). With various experimental protocols and target organisms, these studies produced inconsistent ClO_2 immersion results, ranging from no effect to a >3-log reduction, for microorganisms on produce. In previous investigations, the efficacy of aqueous ClO_2 has been compared with that of other common sanitizers (19, 22, 27). The purpose of the current study was to specifically investigate the sanitizing effects of a ClO_2 solution on *S. enterica* and *E. carotovora* in water, on tomato surfaces, and between tomato loads. Because microbial contaminants on fruit surfaces can be encountered as new contaminants or later in a dehydrated state, the effects of ClO_2 sanitizing treatment on both types of contamination were evaluated.

MATERIALS AND METHODS

Inoculum preparation. Three serovars of *S. enterica* (*Salmonella* Enteritidis ATCC 13076, *Salmonella* Newport ATCC 6962, and *Salmonella* Typhimurium ATCC 14028) and three strains of *E. carotovora* (ATCC 495, ATCC 15359, and ATCC 25272) were maintained at 4°C on tryptic soy agar (TSA) (unless otherwise stated, all media were from Bacto, Becton Dickinson, Sparks, Md.). The cultures were transferred to tryptic soy broth containing 0.6% yeast extract (TSBYE) and incubated for 22 to 24 h at 35 and 28°C for *Salmonella* and *Erwinia*, respectively. The three *Salmonella* or *Erwinia* cultures were then pooled, centrifuged, and resuspended in autoclaved tap water to desired concentrations for immediate use (27).

Chlorine dioxide preparation. Aqueous chlorine dioxide was obtained by adding sterile tap water (2 liters at 23°C) to a commercially available 500-ppm ClO_2 generating pouch (Selectocide-2L500, Selective Micro Technologies, Beverly, Mass.) the day before application. The solution was then diluted to 20, 10, or 5 ppm with sterile tap water (pH 7.9). Addition of ClO_2 caused a minor shift in pH of the water to approximately 7.5. Concentrations of diluted ClO_2 were tested immediately before application with low-range (0, 1, 3, 5, and 10 ppm) chlorine dioxide test

strips provided by the manufacturer (Selective Micro Technologies) (28).

Water system study. Sterile test tubes containing 30 ml of aqueous ClO_2 or water as a control at 23°C were spiked with one of the inocula to obtain approximately 7 log CFU/ml *S. enterica* or *E. carotovora*. After 2, 4, 6, or 10 s, the entire solution was neutralized by mixing with an equal amount (30 ml) of TSBYE containing 0.2% sodium thiosulfate (Fisher Scientific, Fair Lawn, N.J.) before plating (5, 30).

Fruit surface study. Untreated (no washing, oiling, or waxing) Roma tomatoes were obtained directly from a commercial packinghouse (Immokalee, Fla.), stored at 10°C, and used within 3 weeks. The tomatoes were placed at ambient temperature, and a smooth non-stem-scar area (10 cm²) on each tomato was marked with a circle. Each tomato was then spot inoculated with 300 µl of inoculum applied as 20 droplets in the circled area. Inoculated tomatoes were used immediately for a wet-inoculum trial to simulate newly introduced water contamination or were dried at 25°C for 2 h in a forced-air chamber (model 680A, Lab-Line, Dubuque, Iowa) and then held at 23°C and 40 to 50% relative humidity for another 22 h for a dry-inoculum trial to simulate dehydrated contaminants. For each treatment, nine inoculated tomatoes were immersed in 2 liters of ClO_2 or water for 20 to 60 s, and then thin pieces were aseptically cut from the circled surface areas of three treated tomatoes at 20-s intervals. The cut pieces were immediately placed in a sample bag containing 10 ml of TSBYE with 0.2% sodium thiosulfate and macerated in a laboratory blender (IUL Instruments, Barcelona, Spain) at high speed for 4 min before plating.

Cross-contamination study. Chlorine dioxide solution (5 ppm) or water used for the 60-s treatment of the fruit surface study was poured into a second container to immerse nine circle-marked uninoculated tomatoes. After 1 min, the tomatoes were separated into three equal groups. Samples were taken from one group by cutting as described above. The other two groups of tomatoes were immersed in a new batch of either ClO_2 (5 ppm) or water for an additional 1 min before samples were collected. Water samples were obtained before and after each step of the immersion treatment.

Microbial enumeration. Appropriate dilutions of each water system sample (in 0.1% peptone water) were spread plated on TSA and incubated for 48 h at 36 or 28°C for enumeration of *S. enterica* and *E. carotovora*, respectively. In the tomato surface and cross-contamination studies, pathogens were enumerated on selective plating media to exclude background microflora. For *Salmonella*, appropriate dilutions of each sample were spread plated on TSA and incubated at room temperature for 2 h. This culture was overlaid with xylose-lysine-desoxycholate agar (XLD) and further incubated at 36°C (21). Black colonies on TSA-XLD were counted after 24 and 48 h of incubation because no appropriate colonies were found in preliminary tests with noninoculated samples. Representative colonies were biochemically confirmed as *Salmonella* using API 20E test strips (bioMérieux, Hazelwood, Mo.). For *Erwinia*, appropriate dilutions of each sample were spread plated on crystal violet pectate medium (CVP) (12) and incubated at 28°C. Typical colonies forming deep cavities were counted as *Erwinia* after 24 and 48 h of incubation because no other similar colonies were observed in the noninoculated samples. Based on the amount of sample plated, the lowest detection level was 10 CFU/ml for the water system study, 10 CFU/cm² for the fruit surface study, and 1 CFU/ml or 1 CFU/cm² for the cross-contamination study.

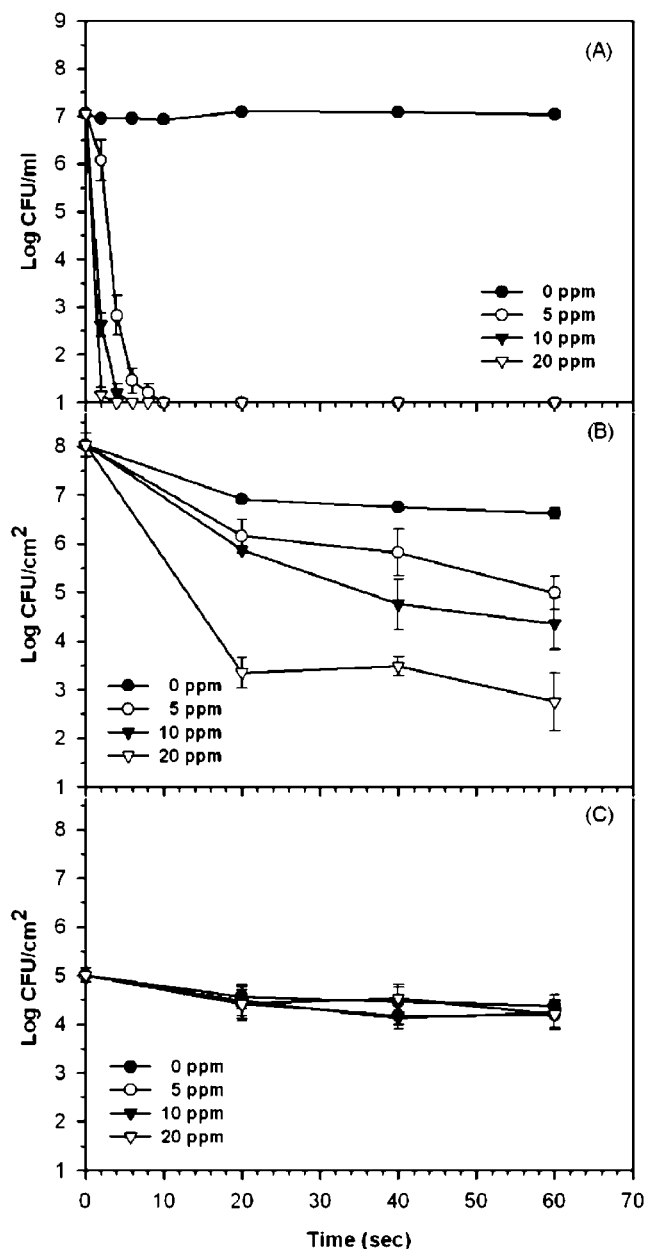


FIGURE 1. Effect of chlorine dioxide concentration on the recovery of *Salmonella enterica* from (A) water (TSA plates), (B) tomato surfaces with wet inoculum (TSA-XLD plates), and (C) tomato surfaces with dry inoculum (TSA-XLD plates).

Statistical analysis. Based on a minimum of 3 replications per treatment, microbial populations were analyzed by *t* test or one-way analysis of variance using SigmaStat software (version 3.0, SPSS Inc., Chicago, Ill.). Differences were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Reducing pathogens in water. As expected, ClO₂ had excellent sanitizing activity against *S. enterica* and *E. carotovora* in water. In autoclaved tap water, 5, 10, and 20 ppm ClO₂ reduced *S. enterica* populations from 7.1 ± 0.1 log CFU/ml to the minimum detection level (10 CFU/ml) after about 10, 6, and 4 s, respectively (Fig. 1A). The same ClO₂ concentrations achieved 5-log reductions of *S. enterica* in 6, 4, and 2 s, respectively. For *E. carotovora*, only

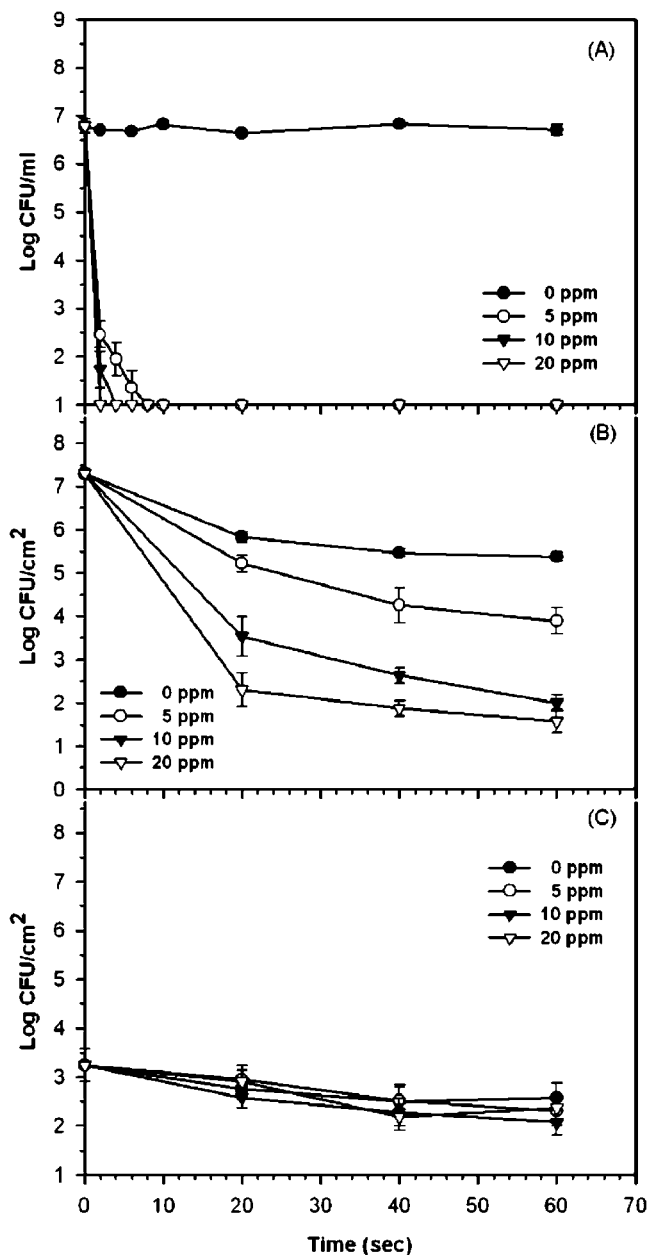


FIGURE 2. Effect of chlorine dioxide concentration on the recovery of *Erwinia carotovora* from (A) water (TSA plates), (B) tomato surfaces with wet inoculum (CVP plates), and (C) tomato surfaces with dry inoculum (CVP plates).

8, 4, and 2 s of exposure to 5, 10, and 20 ppm ClO₂, respectively, were required to reduce the initial population of 6.8 ± 0.0 log CFU/ml to concentrations at or below the level of detection (Fig. 2A). A 5-log reduction of *E. carotovora* was achieved in only 2 s with 10 ppm of ClO₂. Significant reductions in concentrations of *S. enterica* and *E. carotovora* were not observed in the water controls (without ClO₂). Previous investigations have established that untreated water used for fruit farm and packing operations can be a vehicle for introduction of serious pathogens such as *Salmonella* and *Erwinia* onto produce (6, 18). Therefore, use of approved disinfectants, including ClO₂, with clean or potable water in agricultural production and

TABLE 1. Effect of chlorine dioxide immersion in reducing pathogens on the surface of inoculated tomatoes and in dumping water^a

ClO ₂ treatment ^b	<i>Salmonella enterica</i> (log CFU)				<i>Erwinia carotovora</i> (log CFU)			
	Wet inoculum		Dry inoculum		Wet inoculum		Dry inoculum	
	Fruit (per cm ²)	Water (per ml)	Fruit (per cm ²)	Water (per ml)	Fruit (per cm ²)	Water (per ml)	Fruit (per cm ²)	Water (per ml)
None	8.0 ± 0.2 A	≤0.0 ± 0.0 B	5.0 ± 0.1 A	≤0.0 ± 0.0 B	7.3 ± 0.1 A	≤0.0 ± 0.0 B	3.2 ± 0.3 A	≤0.0 ± 0.0 B
0 ppm	6.6 ± 0.1 B	6.7 ± 0.0 A	4.4 ± 0.1 A	4.0 ± 0.3 A	5.4 ± 0.1 B	5.5 ± 0.1 A	2.6 ± 0.3 A	2.3 ± 0.1 A
5 ppm	5.0 ± 0.3 C	0.8 ± 0.3 C	4.2 ± 0.3 A	0.6 ± 0.3 B	3.9 ± 0.3 C	≤0.0 ± 0.0 B	2.3 ± 0.3 A	≤0.0 ± 0.0 B

^a Values are mean ± standard error ($n \geq 3$). Within each column, means followed by different letters are significantly different ($P < 0.05$). Selective plates (TSA-XLD for *Salmonella* and CVP for *Erwinia*) were used for pathogen enumeration.

^b Inoculated tomatoes (one load; nine fruits per treatment) were immersed for 1 min in 2 liters of water (25°C) with or without ClO₂ (5 ppm).

processing systems is recommended to control the risk of contamination associated with waterborne pathogens.

Reducing pathogens on fruit. Whereas ClO₂ concentrations between 5 to 20 ppm killed inoculated *S. enterica* and *E. carotovora* in water almost instantly, the sanitizing effects were reduced on tomatoes. A full minute of contact with ClO₂ at 20 and 10 ppm was required to achieve a 5-log reduction of *S. enterica* and *E. carotovora* on freshly spot-inoculated tomatoes, as indicated by cultures grown on TSA-XLD and CVP, respectively (Figs. 1B and 2B). Immersing wet-inoculated tomatoes in water (0 ppm ClO₂) for 1 min alone reduced *S. enterica* and *E. carotovora* by about 1.2 and 1.9 log CFU/cm², respectively. On tomato surfaces, pathogen concentrations decreased more than 3 log CFU/cm² during 24 h of desiccation at 24 ± 1°C. When compared with pathogen concentrations in water controls without ClO₂, the concentrations of air-dried *Salmonella* and *Erwinia* were not significantly changed by exposure to ≤20 ppm ClO₂ after 1 min (Figs. 1C and 2C). Nevertheless, immersion of dry-inoculated tomatoes in water, with or without the presence of ClO₂, resulted in significant reductions in *Salmonella* and *Erwinia*.

Raw tomatoes can become contaminated with pathogens during production, harvesting, and packing operations. At each of these steps, either natural or human-associated environmental factors may influence the types of microbial contaminants on the product. For example, contaminated runoff from rain, irrigation, or dumping water may deliver initial wet contaminants to produce surfaces. These contaminants then may become dehydrated naturally or artificially during storage. The results of this study suggest that ClO₂ at its currently permitted concentration (5 to 10 ppm) provides significant sanitizing activity to tomatoes only for fruit with freshly introduced waterborne contaminants. Once the contaminants are dried, a brief (≤1 min) ClO₂ bath offers little or no sanitizing effect in comparison to regular tap water.

Preventing cross-contamination. Both the wet and dry inocula of the two pathogens contaminated the immersion water in the absence of an effective level of ClO₂. Immersion of fruit with dried inocula of *Salmonella* at 5.0 log CFU/cm² or *Erwinia* at 3.2 log CFU/cm² for 1 min resulted in pathogen populations of 4.0 and 2.3 CFU/ml, respectively, in the water (Table 1). These pathogens in the

contaminated water could, in turn, cross-contaminate a subsequent load of clean tomatoes and water without ClO₂. For example, immersion of uninoculated tomatoes in water contaminated with *Salmonella* at 6.7 log CFU/ml or *Erwinia* at 5.5 log CFU/ml (both from wet-inoculum trials) for 1 min resulted in pathogen populations of 4.1 and 2.8 CFU/cm², respectively, on the product (Table 2). Subsequent immersion of these newly contaminated tomatoes in clean water further spread the respective pathogens to the water at 4.0 and 2.8 log CFU/ml, respectively. Cross-contamination, both from contaminated water to product and from contaminated product to water, was effectively prevented by adding 5 ppm ClO₂ to the water. In the produce industry, failure to use sanitizing treatments such as aqueous ClO₂ could allow transfer of pathogens from wet equipment surfaces or water to uncontaminated product, as demonstrated here.

Major differences were observed between the wet- and dry-inoculum trials. During the 24-h drying period, the physiological state of the pathogen was likely altered so that it became more firmly attached and penetrated deeper into the product, increasing sanitizer resistance (3). Future research should focus on mechanical treatments such as brushing when testing the effects of ClO₂ on dehydrated contaminants on fruit. Mechanical brushing is commonly used in the produce industry, and the results reported here suggest that contaminants liberated from fruits and vegetables will be more susceptible to sanitizers than those attached to produce surfaces. A combination of physical removal and chemical reaction may yield significantly greater pathogen reductions on fruit surfaces than did the immersion treatment used in this study.

Other researchers have reported results similar to ours, i.e., the reduced effectiveness of ClO₂ on surface-attached microflora. For example, Foschino et al. (14) found that the bactericidal activity of aqueous ClO₂ against *E. coli* was compromised when cells were attached to hard surfaces. Costilow et al. (9) found that 2.5 ppm ClO₂ was effective against microorganisms in wash water, but concentrations as high as 105 ppm failed to reduce the microflora on or in cucumbers. Because of this limitation, scientists have been seeking improved methods of ClO₂ application. In many studies, gaseous ClO₂ has been more lethal to microorganisms than has aqueous ClO₂ (15, 27, 30). The combined effects of ClO₂ and other physical interventions (such

TABLE 2. Effect of chlorine dioxide immersion for preventing pathogen cross-contamination through contaminated water to noninoculated tomatoes^a

ClO ₂ treatment ^b	<i>Salmonella enterica</i> (log CFU)				<i>Erwinia carotovora</i>			
	Wet inoculum		Dry inoculum		Wet inoculum		Dry inoculum	
	Water (per ml)	Fruit (per cm ²)	Water (per ml)	Fruit (per cm ²)	Water (per ml)	Fruit (per cm ²)	Water (per ml)	Fruit (per cm ²)
Experiment 1								
None	6.7 ± 0.0 A	≤0.0 ± 0.0 D	4.0 ± 0.3 A	≤0.0 ± 0.0 B	5.5 ± 0.1 A	≤0.0 ± 0.0 C	2.3 ± 0.1 A	≤0.0 ± 0.0 A
0 ppm	6.6 ± 0.1 A	4.1 ± 0.1 A	4.3 ± 0.1 A	1.3 ± 0.2 C	5.8 ± 0.2 A	2.8 ± 0.2 A	2.5 ± 0.0 A	≤0.0 ± 0.0 A
0 ppm + 0 ppm	4.0 ± 0.0 B	2.7 ± 0.1 B	0.8 ± 0.4 B	0.2 ± 0.3 B	2.8 ± 0.4 B	1.7 ± 0.2 B	≤0.0 ± 0.0 B	≤0.0 ± 0.0 A
0 ppm + 5 ppm	≤0.0 ± 0.0 D	0.6 ± 0.4 C	≤0.0 ± 0.0 B	≤0.0 ± 0.0 B	≤0.0 ± 0.0 C	0.4 ± 0.4 C	≤0.0 ± 0.0 B	≤0.0 ± 0.0 A
Experiment 2								
None	0.8 ± 0.3 C	≤0.0 ± 0.0 D	0.6 ± 0.3 B	≤0.0 ± 0.0 B	≤0.0 ± 0.0 C	≤0.0 ± 0.0 C	≤0.0 ± 0.0 B	≤0.0 ± 0.0 A
5 ppm	≤0.0 ± 0.0 D	≤0.0 ± 0.0 D	≤0.0 ± 0.0 B	≤0.0 ± 0.0 B	≤0.0 ± 0.0 C	≤0.0 ± 0.0 C	≤0.0 ± 0.0 B	≤0.0 ± 0.0 A
5 ppm + 0 ppm	≤0.0 ± 0.0 D	≤0.0 ± 0.0 D	≤0.0 ± 0.0 B	≤0.0 ± 0.0 B	≤0.0 ± 0.0 C	≤0.0 ± 0.0 C	≤0.0 ± 0.0 B	≤0.0 ± 0.0 A
5 ppm + 5 ppm	≤0.0 ± 0.0 D	≤0.0 ± 0.0 D	≤0.0 ± 0.0 B	≤0.0 ± 0.0 B	≤0.0 ± 0.0 C	≤0.0 ± 0.0 C	≤0.0 ± 0.0 B	≤0.0 ± 0.0 A

^a Values are mean ± standard error (*n* ≥ 3). Within each column, means followed by different letters are significantly different (*P* < 0.05). Selective plates (TSA-XLD for *Salmonella* and CVP for *Erwinia*) were used for pathogen enumeration.

^b Contaminated water resulted from immersion of inoculated tomatoes (with either wet or dry inocula) into 0 or 5 ppm ClO₂ (Table 1). For single-immersion treatments (0 or 5 ppm), noninoculated tomatoes (nine fruits per treatment) were immersed for 1 min in 2 liters of the contaminated water. For double-immersion treatments (0 ppm + 0 ppm, 5 ppm + 0 ppm, etc.), the tomatoes from the single-immersion treatments were further immersed (three fruits per treatment) in 667 ml of sterilized water (second batch, 25°C) with or without ClO₂ for 1 min.

as ultrasonication) or chemical sanitizers have been investigated (16, 31). In one recent study, there was a difference between using CVP and a nonselective medium for recovering *Erwinia* cells injured by mild acid treatment (17). Although ClO₂-induced cell injury and subsequent infectivity and recovery issues have not been thoroughly addressed, the potential for lack of growth of sublethally injured *Erwinia* cells on CVP plates should be considered when interpreting the results of the current and previous studies.

In this study, the sanitizing effects of ClO₂ against bacterial pathogens in water and on both wet- and dry-inoculated tomatoes were drastically different. Contamination with human and plant pathogens during fruit handling is best prevented by using an effective disinfectant. After a load of fruit is contaminated, even a proven disinfectant such as ClO₂ will not be fully effective in eliminating such contaminants, particularly when they are in a dehydrated state on the product. The efficacy of aqueous ClO₂ observed in this laboratory-scale study must be validated on an individual basis for commercial operations, particularly those utilizing recycled water that can contain high concentrations of organic or inorganic matter.

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