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Comparison of Chlorine and a Prototype Produce Wash Product for Effectiveness in Killing *Salmonella* and *Escherichia coli* O157:H7 on Alfalfa Seeds

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ABSTRACT

Outbreaks of *Salmonella* and *Escherichia coli* O157:H7 infections associated with alfalfa and other seed sprouts have occurred with increased frequency in recent years. This study was undertaken to determine the efficacy of a liquid prototype produce wash product (Fit), compared with water and chlorinated water, in killing *Salmonella* and *E. coli* O157:H7 inoculated onto alfalfa seeds. We investigated the efficacy of treatments as influenced by seeds from two different lots obtained from two seeds suppliers and by two methods of inoculation. The efficacy of treatments was influenced by differences in seed lots and amount of organic material in the inoculum. Significant ($\alpha = 0.05$) reductions in *Salmonella* populations on seeds treated with 20,000 ppm of chlorine or Fit for 30 min ranged from 2.3 to 2.5 $\log_{10}$ CFU/g and 1.7 to 2.3 $\log_{10}$ CFU/g, respectively. Reductions ($\alpha = 0.05$) in *E. coli* O157:H7 ranged from 2.0 to 2.1 $\log_{10}$ CFU/g and 1.7 to more than 5.4 $\log_{10}$ CFU/g of seeds treated, respectively, with 20,000 ppm of chlorine or Fit. Compared with treatment with 200 ppm of chlorine, treatment with either 20,000 ppm of chlorine or Fit resulted in significantly higher reductions in populations of *Salmonella* and *E. coli* O157:H7. None of the treatments eliminated these pathogens as evidenced by their detection on enrichment of treated seeds. Considering the human health and environmental hazards associated with the use of 20,000 ppm of chlorine, Fit provides an effective alternative to chlorine as a treatment to significantly reduce bacterial pathogens that have been associated with alfalfa seeds.

The first reported outbreak of human illness associated with seed sprouts was in 1973 *(14)*. Soy, mustard, and cress sprouts produced using a home sprouting kit and submitted for microbiological analysis by a person with gastrointestinal illness were found to contain large numbers of aerobic spore–forming bacteria. Bacteriologic examination of seeds from unopened sprouting kits revealed that they contained *Bacillus cereus*. During seed germination, *B. cereus* proliferated to more than 10^7 CFU/g of sprouts. From 1988 to mid-1999, 11 outbreaks of salmonellosis involving 14 *Salmonella* serotypes were linked to sprouts *(11, 17)*. Nine of these outbreaks, as well as two of three outbreaks of *Escherichia coli* O157:H7 infection in 1996 and 1997, were attributed to alfalfa sprouts. A fourth outbreak caused by *E. coli* O157:NM in 1998 was linked to clover and alfalfa sprouts. The largest outbreak of *E. coli* O157:H7 infection reported to date, which occurred in Japan in 1996 *(10)*, involved approximately 6,000 people and was epidemiologically linked to white diakon radish sprouts.

In several outbreaks of *Salmonella* and *E. coli* O157:H7 infections, epidemiologic investigations revealed that sprouts from two or more separate growing facilities were produced from a common lot of seeds *(1, 5, 6, 9, 10, 13, 18)*. Populations of *Salmonella (8)* and *E. coli* O157:H7 *(7, 15)* exceeding 10^6 CFU/g are known to occur on alfalfa sprouts produced from contaminated seeds, and attempts to disinfect these sprouts by treatment with chemicals have been largely ineffective *(15)*. Treatment of radish sprouts with 4,000 ppm of calcinated calcium, on the other hand, has been reported to inhibit growth or inactivate *E. coli* O157:H7 *(2)*. Treatment of seeds, rather than sprouts, for the purpose of killing pathogenic bacteria that may be present would be the most logical intervention to reduce the likelihood of high numbers of pathogenic bacteria developing during sprout production, assuming sprouts are grown using good manufacturing practices.

The effectiveness of chemicals in killing pathogenic bacteria on seeds has been investigated by several researchers. Jaquette et al. *(8)* tested chlorine for its effectiveness in killing *Salmonella* Stanley inoculated onto alfalfa seeds at populations of 10^1 to 10^2 CFU/g. Populations were reduced to less than 1 CFU/g after seeds were treated with 2,040 ppm of free chlorine. In another study *(3)*, treatment with 2,000 ppm of chlorine, 6% H_2O_2, or 80% ethanol reduced *Salmonella* populations on alfalfa seeds by more than 3 logs but did not eliminate the pathogen. Taormina and Beuchat *(16)* studied the efficacy of chemical treatments in eliminating 2.0 to 3.2 $\log_{10}$ *E. coli* O157:H7 per g of alfalfa seeds. Significant ($\alpha = 0.05$) reductions in population were observed after treatment with 500 or 1,000 ppm of chlorine (as $Ca[OCl]_2$) for 3 min but not 10 min and with 2,000

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ppm of $Ca(OCl)_2$, regardless of pretreatment of seeds with a surfactant. Populations were reduced by treating seeds with 30% or 70% ethanol for 3 or 10 min, although germination percentage also markedly decreased. Treatment with up to 8% H_2O_2 significantly reduced populations of *E. coli* O157:H7 on alfalfa seeds, but the pathogen was detected by enrichment.

Treatment of alfalfa seeds containing 2.7 $\log_{10}$ CFU of *E. coli* O157:H7 per g with 20,000 ppm of active chlorine (as $Ca[OCl]_2$) for 3 min has been shown to reduce the population to less than 2 CFU/g; however, the pathogen could be detected by enrichment *(16)*. Based on these observations, the U.S. Food and Drug Administration and the U.S. Environmental Protection Agency jointly worked to establish, in November 1998, a nationwide special exemption for the use of 20,000 ppm $Ca(OCl)_2$ to sanitize seeds intended for sprout production. The National Advisory Committee on Microbiological Criteria for Food *(11)* recommends that seeds intended for sprouts should be subjected to one or more treatments, including 20,000 ppm of $Ca(OCl)_2$, that can effectively reduce or eliminate pathogenic bacteria.

The use of 20,000 ppm of $Ca(OCl)_2$ to sanitize seeds brings with it some hazards to workers and the environment *(4)*. Although numerous other chemical sanitizers have been evaluated as alternatives to chlorine to kill pathogens on alfalfa seeds *(3, 8, 15, 16)*, none has been demonstrated to be completely effective. The study reported herein was undertaken to determine the efficacy of a liquid prototype produce wash product (Fit, Procter and Gamble Co., Cincinnati, Ohio), compared with 200 and 20,000 ppm of chlorine, in killing *Salmonella* and *E. coli* O157:H7 inoculated onto alfalfa seeds. The efficacy of treatments as influenced by different lots of seeds and methods of inoculation was investigated.

MATERIALS AND METHODS

Strains used. Six serotypes of *Salmonella* were used: Agona (alfalfa sprout–associated outbreak), Enteritidis (human feces), Gaminara (orange juice), Michigan (cantaloupe-associated outbreak), Montevideo (raw tomato–associated outbreak), and Typhimurium (bovine feces). Five enterohemorrhagic strains of *E. coli* O157:H7 were studied: 932 (human feces), 994 (salami), E0018 (calf feces), H1730 (lettuce-associated outbreak), and F4546 (alfalfa sprout-associated outbreak).

Laboratory stock cultures of *Salmonella* and *E. coli* O157:H7 stored at 4°C on tryptic soy agar (TSA, pH 7.3) (Difco Laboratories, Detroit, Mich.) were inoculated into 10 ml of tryptic soy broth (TSB, pH 7.3) (Difco), then adapted to grow at 37°C in TSB containing 50 μg/ml of nalidixic acid (TSBN; Sigma Chemical Co., St. Louis, Mo.) by increasing the concentration of the acid in 10-μg/ml increments at successive 24-h transfers during a 10-day period.

Test for cross-strain inhibition. Each strain of test pathogen was examined for its ability to inhibit the growth of all other test strains. Pathogens were grown as described in protocols for preparing 24-h cultures for inoculating seeds (see below). Cultures of all strains were cross-streaked on TSA and incubated at 37°C for 24 h. Plates were examined for inhibition of growth at junctions of cross-streaks.

Preparation of inocula and procedures for inoculation. Two procedures were used to prepare inocula and inoculate alfalfa seeds obtained from two suppliers. The goal was to compare the effectiveness of chemical treatments in killing *Salmonella* and *E. coli* O157:H7 on alfalfa seeds that originated from different lots and to determine if effectiveness is influenced by the type and amount of organic material in the cell inoculum that may remain as residual material on seeds after inoculation and drying.

In procedure 1, alfalfa seeds obtained from supplier A were used. After three successive 24-h loop transfers of *Salmonella* and *E. coli* O157:H7 grown in 10 ml of TSBN at 37°C, 6 ml of culture of each serotype of *Salmonella* or strain of *E. coli* O157:H7 was combined with 1 liter of sterile deionized water at 22 ± 2°C and mixed. Alfalfa seeds (1 kg, 22 ± 2°C) were added to the cell suspension and gently mixed for 1 min. The cell suspension was decanted, and seeds were placed on a double layer of cheesecloth on a wire screen. The screen was positioned on supports 8 cm above the working surface in a biosafety laminar flow hood to facilitate air movement around the seeds for 48 to 50 h. Seeds were mixed occasionally during the drying process.

In procedure 2, alfalfa seeds obtained from supplier B were inoculated with *Salmonella* or *E. coli* O157:H7. *Salmonella* and *E. coli* O157:H7 were grown in 10 ml of TSBN as described in procedure 1. Cultures were transferred twice at 24-h intervals to 100 ml of TSBN. Cultures (100 ml, 24 h, 37°C) of six serotypes of *Salmonella* were combined in a sterile 2-liter beaker. A mixture of five strains of *E. coli* O157:H7 was likewise prepared. Into each mixed-serotype or mixed-strain cell suspension (22 ± 2°C), 500 g of alfalfa seeds was added. After mixing for 30 s, seeds were dried as described in procedure 1.

Regardless of the procedure used for inoculation, dry seeds were stored at 5°C for 1 to 3 weeks before subjecting them to chemical treatments.

Chemical treatments. Alfalfa seeds (10 g) inoculated with *Salmonella* or *E. coli* O157:H7 using procedures 1 and 2 were combined with 40 ml of the following treatments in 250-ml Erlenmeyer flasks: sterile deionized water (control), sterile Dey-Engley (DE) neutralizing broth (Difco) (control), 200 ppm of chlorine solution, 20,000 ppm of chlorine solution (as $Ca[OCl]_2$ in 0.05 M potassium phosphate buffer, pH 7.0), or a liquid prototype produce wash product (Fit), which is an alkaline solution consisting of generally recognized as safe ingredients (water, oleic acid, glycerol, ethanol, potassium hydroxide, sodium bicarbonate, citric acid, and distilled grapefruit oil). The temperature of seeds and control and treatment solutions was 22 ± 2°C. The mixture of seeds and water, DE broth, or chemical solution in a beaker was placed on a rotary shaker (100 rpm) for 15 or 30 min.

Microbiological analysis. Immediately after treatment for 15 or 30 min, decanted undiluted control and treatment solutions (quadruplicate 0.25-ml samples and duplicate 0.1-ml samples) and duplicate samples (0.1 ml) serially diluted in sterile 0.1% peptone were immediately surface plated on appropriate selective media. DE broth (20 ml) was added to wash the seeds, and the mixture was pummeled in a stomacher for 30 s at medium speed. Samples of DE wash broth were also surface plated on selective media.

Samples of control (water or DE broth) and treatment solutions, as well as DE wash broth from seeds inoculated with *Salmonella* using inoculation procedure 1, were plated on bismuth sulfite agar (Difco) supplemented with 50 μg/ml of nalidixic acid (BSAN) and TSA supplemented with 50 μg/ml of nalidixic acid (TSAN). Samples from seeds inoculated with *E. coli* O157:H7 were plated on sorbitol MacConkey agar (Oxoid, Blasingstoke, England) supplemented with 50 μg/ml of nalidixic acid (SMACN)

and TSAN. For seeds inoculated using procedure 2, samples of control and treatment solutions from seeds inoculated with *Salmonella* or *E. coli* O157:H7 were plated only on TSAN. Plates were incubated at 37°C for 24 to 28 h before presumptive colonies of *Salmonella* and *E. coli* O157:H7 were counted. Presumptive colonies were randomly picked and subjected to appropriate biochemical tests for confirmation. Presumptive *E. coli* O157:H7 colonies were tested for latex agglutination (O157) reaction (Oxoid).

Samples of seeds anticipated to contain low numbers of pathogens after treatment were subjected to enrichment procedures. To the mixture of 20 ml of DE broth and 10 g of seeds inoculated with *Salmonella* or *E. coli* O157:H7, respectively, 20 ml of 2× lactose broth (Difco) or 2× modified TSB *(12)* supplemented with 100 μg/ml of nalidixic acid was added. After incubating seeds in lactose broth at 37°C for 24 h, 1 ml was transferred to 10 ml of selenite cystine broth (Difco). Cultures (37°C, 24 h) were streaked on BSAN and again incubated at 37°C for 24 h. Plates were examined for presumptive *Salmonella* colonies. Random colonies were examined for biochemical activities using triple sugar iron agar (Difco) and lysine iron agar (Difco). Cultures from the mixture of seeds and modified nalidixic acid–supplemented TSB incubated at 37°C for 24 h were streaked on SMACN and incubated at 37°C for 24 to 48 h. Presumptive *E. coli* O157:H7 colonies were confirmed using API 20E diagnostic kits (bioMéieux Vitek, Inc., Hazelwood, Mo.) and latex agglutination reaction (Oxoid).

Chlorine analysis. Concentrations of total and free chlorine in hypochlorite solutions were determined with chlorine test kits (Hach Co., Ames, Iowa).

Determination of seed viability. Seeds prepared using the two inoculation procedures and subjected to control and chemical treatments followed by washing with DE broth were tested for germinability. Seeds were rinsed with water by shaking vigorously by hand for 30 s, rather than pummeling in a stomacher, to minimize excess removal of seed coats. Approximately 100 seeds were spread on a water-saturated filter paper in a petri dish. The seeds were covered with a second piece of moistened filter paper and stored at 30°C for 48 to 54 h. Each seed was examined for the development of a radicle, and the percentage of seeds that germinated was calculated.

Statistical analysis. Three replicate experiments for each set of experimental parameters were conducted. Data were subjected to the Statistical Analysis System (SAS Institute, Cary, N.C.) for analysis of variance and Duncan's multiple range tests.

RESULTS AND DISCUSSION

Cross-strain inhibition tests revealed that, within pathogen, none of the strains inhibited the growth of each other. Mixing strains to prepare inocula did not, therefore, present a problem that might be associated with strain interaction.

The results shown in Table 1 are numbers of *Salmonella* recovered on BSAN and TSAN from treated alfalfa seeds inoculated using procedure 1. Differences in numbers of viable cells recovered after 15- or 30-min treatments were minimal. Our inability to recover *Salmonella* from treatment solutions containing 20,000 ppm of chlorine or Fit indicates that cells removed from the seeds, i.e., suspended in treatment solutions, were killed. However, subsequent washing of treated seeds with DE broth removed viable cells that were then detected on recovery media. Fewer cells were recovered on BSAN than on TSAN, indicating that a portion of injured or stressed cells did not resuscitate when plated on the more selective BSAN. Comparison of $\log_{10}$ reductions in the following discussion is based on differences between populations recovered from DE broth used to wash seeds after they were subjected to the water control, DE broth control, or chemical treatments for 30 min. Treatment of inoculated seeds with 200 ppm of chlorine for 30 min resulted in a reduction of 1.9 $\log_{10}$ CFU/g of seeds, whereas treatment with 20,000 ppm of chlorine or Fit produce rinse for 15 or 30 min reduced the population by 2.3 $\log_{10}$, although significant ($P \leq 0.05$) differences among these reductions did not exist. Observations on effectiveness of the 200-ppm treatment are in agreement with another study *(3)* using *Salmonella* Stanley, which showed that treatment of alfalfa seeds with 160 or 390 ppm of chlorine for 30 s caused, respectively, 1.6- and 1.8-$\log_{10}$ CFU/g reductions in viable cells. Neither of these studies are in agreement with a third study *(8)*, also done in our laboratory, which showed that treatment of alfalfa seeds with 100 or 290 ppm of chlorine reduced populations of *Salmonella* Stanley by only 0.3 or 0.6 $\log_{10}$ CFU/g, respectively; treatment with 1,010 ppm of chlorine caused only a 1-$\log_{10}$ CFU/g reduction. Differences in effectiveness of chlorine in the three studies are attributed to the use of different lots of seed, serotypes of *Salmonella,* and procedures for inoculation, treatment, and recovery. All of these factors, separately or in combination, can influence the effectiveness of chlorine and other sanitizers in killing *Salmonella* and other microorganisms on alfalfa seeds.

Also listed in Table 1 are seed germination percentages as influenced by treatment. Exposure of seeds to 20,000 ppm of chlorine for 15 or 30 min or Fit for 30 min resulted in a significant ($\alpha = 0.05$) reduction in the percentage of seeds capable of germination. Seed germination percentages using these treatment parameters, however, were not significantly different. The sensitivity of alfalfa seeds to treatment with 20,000 ppm of chlorine may differ depending on various factors, e.g., cultivar and whether the seed scarified or not. Taormina and Beuchat *(16)* observed that treatment of one lot of seeds with 20,000 ppm of chlorine for 10 min significantly reduced germination percentage, whereas the same treatment of seeds from a second lot was without significant effect on germination. Unfortunately, our supply of alfalfa seeds from supplier B was depleted before germination studies were done, so we do not have germination data to compare with those from seeds obtained from supplier A (Table 1).

The results shown in Table 2 are numbers of *E. coli* O157:H7 recovered on SMACN and TSAN from treated seeds. Procedure 1 was also used to inoculate seeds used in this study. As with *Salmonella* (Table 1), a portion of injured or stressed *E. coli* O157:H7 cells did not repair and form colonies when plated on a selective medium. Counts were higher on TSAN than on SMACN. Also in agreement with observations on *Salmonella,* viable cells of *E. coli* O157:H7 were not detected in solutions of 20,000 ppm of chlorine and Fit after treatment of seeds. *E. coli* O157:H7 was detected in DE broth used to wash seeds treated with 200 or 20,000 ppm of chlorine but not in DE broth used

TABLE 1. *Number of* Salmonella *recovered from treated alfalfa seeds on BSAN and TSAN*[a]

Recovery medium	Control/ treatment	Soak time (min)	Population (log_{10} CFU/g of seed) recovered from[b]: Control/ treatment solution	DE wash broth	Reduction (log_{10} CFU/g of seeds) Control/ treatment solution[c]	DE wash broth[c]	Germination (%)[d]
BSAN	Water	15	6.54 A	6.20 B			95.7 A
		30	6.56 A	6.26 A			93.3 A
	DE broth	15	6.46 A	5.94 AB	0.1	0.3	94.0 A
		30	6.50 A	6.10 AB	0.1	0.2	95.3 A
	Chlorine (200 ppm)	15	4.81 B	4.37 C	1.7	1.8	94.3 A
		30	4.84 B	4.29 C	1.7	2.0	94.3 A
	Chlorine (20,000 ppm)	15	—[e]	3.76 D	>6.5	2.4	86.7 C
		30	—	3.94 D	>6.5	2.3	85.7 C
	Fit	15	—	3.32 E	>6.5	2.9	92.7 AB
		30	—	3.64 D	>6.5	2.6	87.7 BC
TSAN	Water	15	6.30 A	6.14 A			
		30	6.49 A	6.39 A			
	DE broth	15	6.43 A	5.98 A	0	0.1	
		30	6.51 A	6.40 A	0	0	
	Chlorine (200 ppm)	15	4.57 B	4.40 B	1.7	1.7	
		30	4.66 B	4.45 B	1.8	1.9	
	Chlorine (20,000 ppm)	15	—	3.88 BC	>6.3	2.3	
		30	—	4.13 BC	>6.4	2.3	
	Fit	15	—	3.81 C	>6.3	2.3	
		30	—	4.09 BC	>6.4	2.3	

[a] Procedure 1 was used to inoculate seeds. Bismuth sulfite agar with 50 μg/ml of nalidixic acid (BSAN) and tryptic soy agar with 50 μg/ml of nalidixic acid (TSAN) were used to enumerate *Salmonella*; Dey-Engley (DE) broth was used as a control solution for washing seeds and as a neutralizer solution after treatment of seeds.

[b] Mean values in the same column and within the same recovery (enumeration) medium (BSAN or TSAN) that are not followed by the same letter are significantly different ($\alpha = 0.05$). Values obtained from control/treatment solutions and DE wash broth were used to calculate log_{10} CFU/g of seeds.

[c] Within the same recovery medium (BSAN or TSAN), log_{10} reduction compared with respective water control/treatment solution or DE wash solution.

[d] Germination percentage was determined using treated, washed seeds as described in the text. BSAN was not used in the procedure to determine germination percentage; however, for simplicity, data are presented in this table. Mean values that are not followed by the same letter are significantly different ($\alpha = 0.05$).

[e] Less than 1 CFU/0.25 g of seed.

to wash seeds treated with Fit. Enrichment of seeds, however, revealed the presence of *E. coli* O157:H7 in seeds treated with 20,000 ppm of chlorine or Fit. A 2.0-log_{10} CFU/g reduction or a more than 5.4-log_{10} CFU/g reduction in *E. coli* O157:H7 occurred when seeds were treated with 20,000 ppm of chlorine or Fit, respectively. In another study *(16)*, using the same strains of *E. coli* O157:H7 and the same procedure for inoculation, treatment of seeds with 20,000 ppm of chlorine for 10 min resulted in a 2.8-log_{10} CFU/g reduction in population. Different lots of seeds and different procedures for recovering *E. coli* O157:H7 were used, however, which may have influenced the number of surviving cells enumerated in the two studies. Alfalfa seeds are sometimes scarified by seed suppliers to enhance rapid and uniform germination. This process may also facilitate lodging of *E. coli* O157:H7 and *Salmonella* cells on the seed surface, thus resulting in protection against contact with treatment solutions. It is not known if seeds obtained from suppliers A and B used in this study or the previous study *(16)* were scarified. In the study reported herein, however, Fit was more effective than 20,000 ppm of chlorine in killing *E. coli* O157:H7 on alfalfa seeds inoculated using procedure 1.

Results of experiments using procedure 2 to inoculate seeds obtained from supplier B are presented in Table 3. Since higher numbers of *Salmonella* and *E. coli* O157:H7 were recovered on TSAN compared with selective media (Tables 1 and 2), only TSAN was used as an enumeration medium. The absence of viable cells of *Salmonella* and *E. coli* O157:H7 in 20,000 ppm of chlorine and Fit solutions after treatment of seeds was again observed (Table 3). Reduction in populations of *Salmonella* were 0.2, 2.5, and 1.7 log_{10} CFU/g when seeds were treated with 200 ppm of chlorine, 20,000 ppm of chlorine, or Fit, respectively. This compares with 1.9-, 2.3-, and 2.3-log_{10} CFU/g reductions, respectively, using seeds inoculated using procedure 1 (Table 1). Treatment with Fit killed as many or significantly more *Salmonella* than treatment with 200 ppm of chlorine, depending on the inoculation procedure. Likewise, equal or significantly greater reductions in number of *Salmonella* oc-

TABLE 2. *Number of* E. coli *O157:H7 recovered from treated alfalfa seeds on SMCAN and TSAN*[a]

Recovery medium	Control/treatment	Soak time (min)	Population (log_{10} CFU/g of seed) recovered from[b]: Control/treatment solution	Population (log_{10} CFU/g of seed) recovered from[b]: DE wash broth	No. of samples positive by enrichment/no. of examined samples	Reduction (log_{10} CFU/g of seeds)[c]: Control/treatment solution	Reduction (log_{10} CFU/g of seeds)[c]: DE wash broth
SMACN	Water	15	5.12 AB	5.31 A			
		30	5.34 A	5.25 A			
	DE broth	15	4.95 B	4.65 B		0.2	0.7
		30	4.78 B	5.01 AB		0.5	0.2
	Chlorine (200 ppm)	15	4.32 C	3.96 C		0.8	1.4
		30	4.21 C	3.95 C		0.6	1.3
	Chlorine (20,000 ppm)	15	—[d]	2.04 E	1/1	>5.1	3.3
		30	—	3.06 D	2/2	>5.3	2.2
	Fit	15	—	—	3/3	>5.1	>5.3
		30	—	—	3/3	>5.3	>5.2
TSAN	Water	15	5.22 AB	5.42 A			
		30	5.44 A	5.37 A			
	DE broth	15	4.95 B	4.89 A		0.3	0.5
		30	4.88 B	5.25 A		0.6	0.1
	Chlorine (200 ppm)	15	4.11 C	4.08 B		1.1	1.3
		30	4.24 C	4.22 B		1.2	1.2
	Chlorine (20,000 ppm)	15	—	2.78 D	1/1	>5.2	2.6
		30	—	3.39 C	2/2	>5.4	2.0
	Fit	15	—	—	3/3	>5.2	>5.4
		30	—	—	3/3	>5.4	>5.4

[a] Procedure 1 was used to inoculate seeds. Sorbitol MacConkey agar with 50 μg/ml of nalidixic acid (SMACN) and tryptic soy agar with 50 μg/ml of nalidixic acid (TSAN) were used to enumerate *E. coli* O157:H7; Dey-Engley (DE) broth was used as a control solution for washing seeds and as a neutralizer solution after treatment of seeds.

[b] Mean values in the same column and within the same recovery (enumeration) medium that are not followed by the same letter are significantly different ($\alpha = 0.05$). Values obtained from control/treatment solutions and DE wash broth were used to calculate log_{10} CFU/g of seeds.

[c] Within recovery medium (SMACN or TSAN), log_{10} reduction compared with respective water control/treatment solution or DE wash solution.

[d] Less than 1 CFU/0.25 g of seed in the control/treatment solution and less than 1 CFU/0.5 g of seed in the DE wash broth.

curred when seeds were treated with 20,000 ppm of chlorine compared with Fit, depending on the inoculation procedure.

Treatment of seeds with 20,000 ppm of chlorine resulted in equal or a significantly greater reduction in viable *E. coli* O157:H7 compared with treatment with Fit, which, in turn, resulted in a significantly greater reduction compared with treatment with 200 ppm of chlorine (Table 3). The actual difference in number of *E. coli* O157:H7 cells recovered from seeds treated with 20,000 ppm of chlorine and Fit, although significant, was only 0.3 log_{10} CFU/g. The reduced effectiveness of Fit, compared with 20,000 ppm of chlorine, in killing *E. coli* O157:H7 inoculated onto seeds using procedure 2 (Table 3) compared with procedure 1 (Table 2) may be due to the use of different seeds and/or the amount of residual organic material adhering to seeds as a result of different concentrations of organic materials in the inocula. Inoculum used in procedure 2 contained about a 30-fold higher concentration of organic material compared with the inoculum used in procedure 1, which one would expect to result in a higher concentration on the surface of inoculated dry seeds. The presence of the additional organic load would be anticipated to lessen the effectiveness of sanitizers, particularly those such as chlorine that are neutralized on contact with organic material. In combination with other factors, this appears to be the case for 200 ppm of chlorine and Fit treatments but not the 20,000 ppm of chlorine treatment of seeds inoculated with *Salmonella* or *E. coli* O157:H7.

Taormina and Beuchat *(16)* reported that free chlorine in a 200-ppm solution decreased by about 90%, i.e., to about 20 ppm, within 15 min when alfalfa seeds were treated using the same ratio of seeds to solution (1:4, wt/vol) used in the study reported herein, whereas free chlorine in a 20,000-ppm solution decreased by about 20%, i.e., to about 16,000 ppm, using the same protocol. The high residual concentration of free chlorine in the 20,000-ppm treatment solution remaining after contact with seeds coated with different concentrations of foreign organic material (dried TSBN) resulting from the two inoculation procedures (1 and 2) would be expected to result in higher lethality to *Salmonella* and *E. coli* O157:H7 enmeshed in this material.

Considering differences in inoculation procedure and source of alfalfa seeds, it is difficult to predict the effectiveness of 20,000 ppm of chlorine or Fit in killing *Salmonella* or *E. coli* O157:H7. Factors such as seed cultivar,

TABLE 3. *Numbers of* Salmonella *and* E. coli *O157:H7 recovered from treated alfalfa seeds on TSAN*[a]

Pathogen	Control/treatment	Soak time (min)	Population (log_{10} CFU/g of seed) recovered from[b]: Control/ treatment solution	Population (log_{10} CFU/g of seed) recovered from[b]: DE wash broth	Reduction (log_{10} CFU/g of seeds)[c] Control/ treatment solution	Reduction (log_{10} CFU/g of seeds)[c] DE wash broth
Salmonella	Water	15	5.30 C	5.80 CD		
		30	5.70 B	5.92 BC		
	DE broth	15	5.92 B	6.11 A	0	0
		30	6.17 A	5.97 AB	0	0
	Chlorine (200 ppm)	15	2.93 E	5.56 E	2.4	0.2
		30	3.41 D	5.72 DE	2.3	0.2
	Chlorine (20,000 ppm)	15	—[d]	3.73 G	>5.3	2.1
		30	—	3.38 H	>5.7	2.5
	Fit	15	—	4.20 F	>5.3	1.6
		30	—	4.20 F	>5.7	1.7
E. coli O157:H7	Water	15	4.68 B	5.38 C		
		30	4.92 B	5.53 BC		
	DE broth	15	5.75 A	5.69 AB	0	0
		30	5.92 A	5.77 A	0	0
	Chlorine (200 ppm)	15	1.99 D	5.38 C	2.7	0
		30	3.58 C	5.42 C	1.3	0.1
	Chlorine (20,000 ppm)	15	—	3.83 D	>4.6	1.6
		30	—	3.44 E	>4.9	2.1
	Fit	15	—	3.85 D	>4.6	1.5
		30	—	3.86 D	>4.9	1.7

[a] Procedure 2 was used to inoculate seeds. Tryptic soy agar with 50 μg/ml of nalidixic acid (TSAN) was used to enumerate *Salmonella* and *E. coli* O157:H7; Dey-Engley (DE) broth was used as a control solution for washing seeds and as a neutralizer solution after treatment of seeds.

[b] Mean values in the same column and within the pathogen that are not followed by the same letter are significantly different ($P \leq 0.05$). Values obtained from analysis of control/treatment solutions and DE wash broth were used to calculate log_{10} CFU/g of seeds. *E. coli* O157:H7 and *Salmonella* were enumerated on TSAN.

[c] Within each pathogen, log_{10} reduction compared with respective water control/treatment solution or DE wash solution.

[d] Less than 1 CFU/0.25 g of seed.

degree of hardness, age, pretreatment (scarification), and seed coat damage, as well as the type and amount of organic material surrounding the target cells, are likely to influence the adhesion characteristics of cells and the lethal effect of sanitizers. Results of this study do show, however, that differences in alfalfa seeds obtained from two suppliers and differences in the amount of organic material in the inoculum influence the number of cells adhering to inoculated seeds and efficacy of sanitizers. Results also show that similar and significant reductions in populations of *Salmonella* and *E. coli* O157:H7 occur in alfalfa seeds treated with either 20,000 ppm of chlorine or Fit compared with treatment with 200 ppm of chlorine.

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