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Efficacy of Chemical Treatments in Eliminating *Salmonella* and *Escherichia coli* O157:H7 on Scarified and Polished Alfalfa Seeds

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ABSTRACT

Alfalfa seeds are sometimes subjected to a scarification treatment to enhance water uptake, which results in more rapid and uniform germination during sprout production. It has been hypothesized that this mechanical abrasion treatment diminishes the efficacy of chemical treatments used to kill or remove pathogenic bacteria from seeds. A study was done to compare the effectiveness of chlorine (20,000 ppm), H_2O_2 (8%), $Ca(OH)_2$ (1%), $Ca(OH)_2$ (1%) plus Tween 80 (1%), and $Ca(OH)_2$ (1%) plus Span 20 (1%) treatments in killing *Salmonella* and *Escherichia coli* O157:H7 inoculated onto control, scarified, and polished alfalfa seeds obtained from two suppliers. The influence of the presence of organic material in the inoculum carrier on the efficacy of sanitizers was investigated. Overall, treatment with 1% $Ca(OH)_2$ was the most effective in reducing populations of the pathogens. Reduction in populations of pathogens on seeds obtained from supplier 1 indicate that chemical treatments are less efficacious in eliminating the pathogens on scarified seeds compared to control seeds. However, the effectiveness of chemical treatment in removing *Salmonella* and *E. coli* O157:H7 from seeds obtained from supplier 2 was not markedly affected by scarification or polishing. The presence of organic material in the inoculum carrier did not have a marked influence on the efficacy of chemicals in reducing populations of test pathogens. Additional lots of control, scarified, and polished alfalfa seeds of additional varieties need to be tested before conclusions can be drawn concerning the impact of mechanical abrasion on the efficacy of chemical treatment in removing or killing *Salmonella* and *E. coli* O157:H7.

Several outbreaks of *Salmonella* and *Escherichia coli* O157:H7 infections associated with alfalfa sprouts have occurred in the past decade (for reviews, see NACMCF *(7)* and Taormina et al. *(15)*). Contamination of seeds may occur in the field or at any stage of harvesting, storage, or sprout production. Both pathogens can grow to populations exceeding 10^7 CFU/g of sprouts during production and retain high viability during subsequent storage at refrigeration temperature *(3, 4, 14)*.

Researchers have evaluated numerous chemical soak treatments for their efficacy in killing *Salmonella* and *E. coli* O157:H7 on alfalfa seeds *(1–4, 13, 16)*. Hypochlorite, chlorine dioxide, organic acids, hydrogen peroxide, ethanol, trisodium phosphate, calcium hydroxide, and commercial formulations containing generally recognized as safe ingredients have exhibited a range of performance in killing these pathogens inoculated onto alfalfa seeds. To date, a chemical treatment has not been evaluated and validated that eliminates more than 5 $\log_{10}$ CFU of pathogens/g on seeds without compromising seed germination percentage. The lack of effectiveness of chemical disinfectants has been attributed to the inaccessibility of pathogens to the chemical solutions. Cells are thought to lodge in cracks, crevices, and damaged areas on the surface of seeds and between the seed coat and cotyledon, thereby protecting them against contact with lethal components in chemical treatment solutions.

Alfalfa seeds can vary substantially in their ability to imbibe water. Impermeability is caused by cells with thick walls in the outer end of the palisade tissue of the seed coat or by the presence of resinous cuticle on the surface of the seed *(5, 11)*. Differences in the percentage of "hard" seeds are influenced by factors such as seed variety, production location, and postharvest storage conditions *(9, 12)*. Scarification, i.e., mechanical abrasion of the seed coat, reduces the percentage of hard seeds and facilitates more rapid and uniform uptake of water during the soaking and germination stages of sprout production. A polishing process designed to achieve the same objectives, but with less disruption of the seed coat, has been described by Mumm *(6)*. Treatment of alfalfa seeds with microwave and radio frequency electric energy has been shown to increase water permeability of alfalfa seed coats *(8)* but has not been applied to seeds intended for sprout production.

It is hypothesized that disruption of the seed coat cuticle and cotyledon tissue would create areas in which microorganisms could lodge and be less exposed to treatment with chemical sanitizers. The influence of scarification and polishing on uptake of pathogens and subsequent resistance to removal from alfalfa seeds, however, has not been reported. The objective of the study reported here was to determine if the efficacy of chemical sanitizers in removing

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Salmonella and *E. coli* O157:H7 from alfalfa seeds is affected by scarification or polishing before inoculation. The influence of the presence of organic material in the inoculum carrier on the efficacy of sanitizers in removing pathogens from control, scarified, and polished seeds was also investigated.

MATERIALS AND METHODS

Strains of microorganisms. Five serotypes of *Salmonella* were used: Anatum (H3536), Cubana (H7976), Infantis (H3517), and Stanley (H1256), all from alfalfa sprout-associated outbreaks, and Montevideo (G4639), from a tomato-associated outbreak. Five *E. coli* O157:H7 strains were used: 932 (human isolate), 994 (salami isolate), E0018 (calf fecal isolate), H1730 (human isolate from an outbreak associated with lettuce), and F4546 (human isolate from an outbreak associated with alfalfa sprouts).

Seeds. Two lots of alfalfa seeds grown in the northwestern United States or western Canada were obtained from two different sources. Both lots were purported to be varieties characterized by a high percentage of hard seeds. Supplier 1 provided control (nonscarified) seeds (variety not known) and scarified seeds from the same lot; supplier 2 provided control seeds (Algonquin variety) and two sublots of the same seeds that were either scarified or polished. Seeds from both suppliers were held at 5°C until used in experiments.

Preparation of inoculum and enumeration of pathogens. Cells of *Salmonella* and *E. coli* O157:H7 were adapted to grow in tryptic soy broth (pH 7.3, Difco Laboratories, Detroit, Mich.) supplemented with nalidixic acid (50 μg/ml). Cultures incubated at 37°C were transferred (one loopful to 10 ml) three times at 24-h intervals preceding use as inocula. Populations of *Salmonella* in 24-h cultures were determined by surface plating (0.1 ml, in duplicate) serially diluted cultures in sterile 0.1% peptone on bismuth sulfite agar (BSA; Difco) supplemented with nalidixic acid (50 μg/ml) (BSAN) and tryptic soy agar (TSA; Difco) supplemented with nalidixic acid (50 μg/ml) (TSAN). Populations of *E. coli* O157:H7 in 24-h cultures were determined by plating diluted cultures on sorbitol MacConkey agar (SMA; Oxoid, Basingstoke, Hampshire, UK) supplemented with nalidixic acid (50 μg/ml) (SMAN) and TSAN. Cultures were plated on both TSAN and traditional selective agars for the purpose of determining the ability of healthy cells of each pathogen to develop colonies on the two selective media. Inoculated recovery media were incubated at 37°C for 24 h before colonies were counted.

Inoculation of seeds. Sterile 5% horse serum albumen (HSA; Difco) was used as a carrier for the purpose of determining the influence of enmeshing cells in an organic material on the efficacy of chemical treatment in removing them from control, scarified, and polished seeds. Cultures (1.5 ml) of each serotype of *Salmonella* grown at 37°C for 24 h were combined with 250 ml of sterile deionized water or 250 ml of 5% HSA and gently mixed for 30 s. Untreated (control), scarified, and polished seeds (250 g) were added to the diluted cell suspension and gently stirred for 1 min. The seeds were separated from the cell suspension by pouring the mixture over a double layer of cheesecloth supported by a wire screen elevated approximately 5 cm above the work surface of a laminar flow hood. Seeds were spread in a layer approximately 0.5 cm thick and dried at 23 ± 2°C for 72 h. Seeds were stirred each day to enhance uniform drying. Dry seeds (10 g) were placed in Stomacher 80 bags (Seward, London, UK), sealed, and stored at 5°C for at least 7 days before using in experiments to evaluate the effectiveness of chemical treatments. The same procedures were used to inoculate seeds with 24-h cultures of *E. coli* O157:H7.

Preparation of treatment solutions. Previous experiments showed that 1% $Ca(OH)_2$ and 8% H_2O_2 compared favorably with 20,000 ppm chlorine in killing *Salmonella* on alfalfa seeds *(16).* Based on these and other observations, the following chemical solutions were evaluated for their efficacy in killing or removing *Salmonella* and *E. coli* O157:H7 on untreated (control), scarified, and polished seeds: $Ca(OCl)_2$ (20,000 ppm free chlorine, Fisher Chemical, Fair Lawn, N.J.) in 0.05 M potassium phosphate buffer (pH 6.8); H_2O_2 (8%, vol/vol; VWR, West Chester, Pa.); $Ca(OH)_2$ (1%; Sigma Chemical Co., St. Louis, Mo.); $Ca(OH)_2$ (1%) plus Tween 80 (1%; Sigma), and $Ca(OH)_2$ (1%) plus Span 20 (1%) (Rohm and Haas, Philadelphia, Pa.). Sterile deionized water was used as a control. All chemical solutions were prepared no more than 30 min before use. The temperature of the solutions and seeds at the time of treatment was 23 ± 2°C. The concentration of free chlorine in the $Ca(OCl)_2$ solution was determined using an amperometric titrator (Hach, Loveland, Colo.).

Chemical treatment and enumeration of pathogens. Seeds (10 g) inoculated with *Salmonella* or *E. coli* O157:H7 were combined with 40 ml of sterile deionized water (control) or chemical solution in a Stomacher 80 bag and soaked at 23 ± 2°C for 10 min while being agitated on a rotary shaker. After decanting water or treatment solution, 20 ml of sterile Dey-Engley (DE) neutralizing broth (pH 7.6 ± 0.2, Difco) was added to seeds treated with water, 8% H_2O_2, or 20,000 ppm chlorine. DE broth (20 ml) adjusted to pH 6.5 was added to seeds treated with $Ca(OH)_2$, with or without Tween 80 or Span 20. The mixture of DE broth and seeds was pummeled in a stomacher at medium speed for 1 min.

To enumerate *Salmonella* on treated seeds, DE wash broth was surface plated in quadruplicate (0.25 ml) and duplicate (0.1 ml) or serially diluted in sterile 0.1% peptone and plated in duplicate (0.1 ml) on BSAN and TSAN. Presumptive colonies formed on plates incubated at 37°C for 24 h were counted. Colonies were randomly picked and subjected to confirmation using the *Salmonella* latex agglutination assay (Oxoid). After removal of approximately 100 seeds for the purpose of determining germination percentage and samples for plating on BSAN and TSAN, 20 ml of double-strength lactose broth (Difco) supplemented with nalidixic acid (100 μg/ml) was added to the mixture of seeds and DE broth. After incubation at 37°C for 24 h, cultures were streaked on BSAN. Presumptive colonies were randomly chosen for confirmation tests.

Populations of *E. coli* O157:H7 on inoculated seeds treated with chemical solutions as described above for seeds inoculated with *Salmonella* were enumerated by surface plating undiluted and diluted DE broth on SMAN and TSAN. Presumptive *E. coli* O157:H7 colonies formed on plates incubated at 37°C for 24 h were counted; selected colonies were confirmed by O157 latex agglutination reaction (Oxoid) and API 20E diagnostic assay (BioMérieux Vitek, Inc., Hazelwood, Mo.). After removal of approximately 100 seeds from each mixture of seed and DE broth for the purpose of determining germination percentage, 20 ml of double-strength modified tryptic soy broth *(10)* supplemented with nalidixic acid (100 μg/ml) was added to each bag. After incubation for 24 h at 37°C, cultures were streaked on SMAN. Presumptive colonies were confirmed as described above.

Determination of germination percentage. Approximately 100 control or treated seeds were placed between two pieces of water-saturated 90-mm-diameter no. 4 filter paper (Whatman In-

TABLE 1. *Populations of* Salmonella *recovered from control (nonscarified) and scarified alfalfa seeds (supplier 1) following chemical treatment*

Seed condition	Inoculum carrier	Treatment	Population (log_{10} CFU/g)[a]		Reduction (log_{10} CFU/g)[d]	Germination (%)[a]
			BSAN[b]	TSAN[c]		
Control	Water	Water (control)	3.88 A	4.21 A		94 A–C
		Chlorine (20,000 ppm)	<0.30 F	0.40 DE	3.91	95 A–C
		H_2O_2 (8%)	1.05 B–E	1.04 B–D	3.27	97 AB
		$Ca(OH)_2$ (1%)	0.50 D–F	0.56 C–E	3.75	90 A–C
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	0.20 EF	0.45 C–E	3.86	92 A–C
		$Ca(OH)_2$ (1%) + Span 20 (1%)	1.19 B–D	1.25 B–D	3.06	68 D
	HSA (5%)	Water (control)	3.76 A	4.35 A		94 A–C
		Chlorine (20,000 ppm)	0.46 D–F	0.55 C–E	3.80	98 A
		H_2O_2 (8%)	1.44 BC	1.01 B–D	3.34	92 A–C
		$Ca(OH)_2$ (1%)	0.26 D–F	0.26 DE	4.09	90 A–C
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	1.00 B–E	1.22 B–D	3.13	93 A–C
		$Ca(OH)_2$ (1%) + Span 20 (1%)	1.50 B	1.71 B	2.64	94 A–C
Scarified	Water	Water (control)	3.16 A	3.59 A		97 AB
		Chlorine (20,000 ppm)	<0.30 F	<0.30 E[e]	>3.29	98 A
		H_2O_2 (8%)	0.48 D–F	0.30 DE	3.29	96 AB
		$Ca(OH)_2$ (1%)	<0.30 F	<0.30 E[e]	>3.29	84 A–C
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	0.30 D–F	0.46 C–E	3.13	85 A–C
		$Ca(OH)_2$ (1%) + Span 20 (1%)	0.30 D–F	1.08 B–D	2.51	82 BC
	HSA (5%)	Water (control)	3.34 A	3.96 A		98 AB
		Chlorine (20,000 ppm)	0.55 C–F	0.75 B–E	3.21	97 AB
		H_2O_2 (8%)	0.63 B–F	1.08 B–D	2.88	96 A–C
		$Ca(OH)_2$ (1%)	0.10 EF	0.30 DE	3.66	87 A–C
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	0.46 D–F	0.80 B–E	3.16	95 A–C
		$Ca(OH)_2$ (1%) + Span 20 (1%)	0.78 B–F	1.42 BC	2.54	81 C

[a] Values in the same column that are followed by the same letter are not significantly different ($\alpha > 0.05$).
[b] Populations of *Salmonella* recovered on BSA supplemented with 50 μg/ml nalidixic acid.
[c] Populations of *Salmonella* recovered on TSA supplemented with 50 μg/ml nalidixic acid.
[d] Within the same seed condition and inoculum carrier, log_{10} reduction compared with number recovered on TSAN from seeds treated with water (control).
[e] *Salmonella* was detected by enrichment.

ternational Ltd., Maidstone, UK) in a 90-mm-diameter petri dish. Seeds were placed in the dark at 30°C for 3 days, with periodic application of water to maintain a high-moisture environment. The number of germinated seeds was determined, and the percentage that germinated was calculated.

Statistical analysis. All experiments were replicated three times. Data were subjected to SAS (Statistical Analysis Systems Institute, Cary, N.C.) for analysis of variance and Duncan's multiple range tests to determine significant differences ($\alpha = 0.05$) in populations of each pathogen recovered from seeds subjected to various experimental treatments.

RESULTS AND DISCUSSION

Previous studies *(2, 13, 16)* have shown that traditional selective media do not support colony development by a portion of *Salmonella* and *E. coli* O157:H7 exposed to some of the same chemical treatments evaluated in this study. Although nalidixic acid would prevent the growth of most gram-negative bacteria that might be present on alfalfa seeds, the potential for interference by background microbiota of *Salmonella* and *E. coli* O157:H7 on TSAN exists. For these reasons, TSAN and BSAN (for enumeration of *Salmonella*) and TSAN and SMAN (for enumeration of *E. coli* O157:H7) were used in all experiments for enumerating test pathogens on untreated and treated seeds.

Salmonella and *E. coli* were not recovered from uninoculated seeds. Regardless of the source for seeds, seed condition (i.e., control, scarified, or polished), inoculum carrier, or chemical treatment, higher populations of *Salmonella* and *E. coli* O157:H7 were recovered on TSAN than on BSAN or SMAN, respectively. *Salmonella* counts were 0.06 to 0.62 log_{10} CFU/g higher, and *E. coli* O157:H7 counts were 0.32 to 0.87 log_{10} CFU/g higher when samples from seeds treated with water (control) were plated on TSAN compared to traditional selective media. Larger differences in counts on TSAN and selective media were observed in seeds treated with some of the chemical solutions. This is in agreement with other reports describing the poor performance of BSA and BSAN in recovering *Salmonella (16)* and SMA and SMAN in recovering *E. coli* O157:H7 *(2, 4)* from chemically treated alfalfa seeds. Lower counts on highly selective media may be due to the inability of cells that have been injured by desiccation or chemical treatment to resuscitate. In the tables to follow, numbers of *Salmonella* and *E. coli* O157:H7 recovered on TSAN rep-

TABLE 2. *Populations of* E. coli *O157:H7 recovered from control (nonscarified) and scarified alfalfa seeds (supplier 1) following chemical treatment*

Seed condition	Inoculum carrier	Treatment	Population (log_{10} CFU/g)[a]		Reduction (log_{10} CFU/g)[d]	Germination (%)[a]
			BSAN[b]	TSAN[c]		
Control	Water	Water (control)	3.19 A	3.99 A		91 AB
		Chlorine (20,000 ppm)	0.30 D–F	1.46 CD	2.53	95 A
		H_2O_2 (8%)	0.20 D–F	1.09 D–F	2.90	96 A
		$Ca(OH)_2$ (1%)	<0.30 F	0.76 E–G	3.23	83 A–D
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	<0.30 F	0.40 FG	3.59	85 A–C
		$Ca(OH)_2$ (1%) + Span 20 (1%)	0.68 F	1.67 B–D	2.32	96 A
	HSA (5%)	Water (control)	3.24 A	4.11 A		89 A–C
		Chlorine (20,000 ppm)	1.34 B	2.33 B	1.78	94 A
		H_2O_2 (8%)	<0.30 F	1.40 CD	2.71	96 A
		$Ca(OH)_2$ (1%)	0.36 D–F	0.59 FG	3.52	87 A–C
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	<0.30 F	0.50 FG	3.61	67 D
		$Ca(OH)_2$ (1%) + Span 20 (1%)	0.46 D–F	1.05 D–F	3.06	97 A
Scarified	Water	Water (control)	2.95 A	3.68 A		91 AB
		Chlorine (20,000 ppm)	0.56 D–F	1.42 C–E	2.26	93 A
		H_2O_2 (8%)	<0.30 F	1.47 CD	2.21	93 A
		$Ca(OH)_2$ (1%)	<0.30 F	0.40 FG	3.20	84 A–D
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	0.10 EF	0.20 G	3.48	78 B–D
		$Ca(OH)_2$ (1%) + Span 20 (1%)	0.62 DE	1.44 CD	2.24	88 AB
	HSA (5%)	Water (control)	3.33 A	3.99 A		92 A
		Chlorine (20,000 ppm)	1.16 BC	1.95 BC	2.04	93 A
		H_2O_2 (8%)	<0.30 F	1.58 B–D	2.41	98 A
		$Ca(OH)_2$ (1%)	<0.30 F	0.70 FG	3.29	93 A
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	0.10 EF	0.46 FG	3.53	70 CD
		$Ca(OH)_2$ (1%) + Span 20 (1%)	1.29 B	1.91 BC	2.08	86 A–C

[a] Values in the same column that are followed by the same letter are not significantly different ($\alpha = 0.05$).
[b] Populations of *E. coli* O157:H7 recovered on SMA supplemented with 50 μg/ml nalidixic acid.
[c] Populations of *E. coli* O157:H7 recovered on TSA supplemented with 50 μg/ml nalidixic acid.
[d] Within the same seed condition and inoculum carrier, log_{10} reduction compared with number recovered on TSAN from seeds treated with water (control).

resent populations closer to those surviving various treatments.

Seeds from supplier 1. Results of chemical treatments of control and scarified seeds obtained from supplier 1 and inoculated with *Salmonella* are shown in Table 1. Within each water or chemical treatment, regardless of seed condition (control or scarified) or inoculum carrier (water or HSA), there were no significant differences ($\alpha = 0.05$) in the number of *Salmonella* recovered on TSAN. Treatment of seeds for 10 min with 20,000 ppm chlorine, 8% H_2O_2, or 1% $Ca(OH)_2$, with or without 1% Tween 80, reduced populations by 2.88 to 4.09 log_{10} CFU/g. Treatment with 1% $Ca(OH)_2$ plus 1% Span 20 was the least effective among chemical treatments in eliminating *Salmonella* on seeds and, unlike the other test chemicals, tended to reduce seed germination percentage. A comparison of reductions in log_{10} CFU/g reveals that, within inoculum carrier and chemical treatment, the decrease in population compared to the water (control) treatment was generally less on scarified seeds than on control seeds. This would suggest that *Salmonella* populations are more difficult to remove from scarified seeds compared to nonscarified seeds. Although seeds obtained from supplier 1 purportedly were hard seeds, the high germination percentage of control seeds would suggest otherwise. Scarification did not significantly increase the percentage of seeds that germinated within 72 h.

The efficacy of chemical treatment in eliminating *E. coli* O157:H7 inoculated onto seeds obtained from supplier 1 is shown in Table 2. With the exception of seeds treated with 20,000 ppm chlorine or $Ca(OH)_2$ plus 1% Span 20, within each water or chemical treatment, regardless of seed condition or inoculum carrier, there were no significant differences ($\alpha = 0.05$) in the number of *E. coli* O157:H7 recovered on TSAN. However, with the exception of scarified seeds inoculated using HSA as a carrier and treated with 20,000 ppm chlorine or 1% $Ca(OH)_2$ plus 1% Span 20, within inoculum carrier and chemical treatment, the reduction in population compared to water (control) treatment was greater on control seeds compared to scarified seeds, which is consistent with observations on seeds inoculated with *Salmonella.* Chemical treatments resulted in reductions of *E. coli* O157:H7 of 1.78 to 3.61 log_{10} CFU/g of seeds, a range considerably less than that observed for *Salmonella* (Table 1). Overall, treatment with 1% $Ca(OH)_2$, with or without 1% Tween 80, was most effective in eliminating *E. coli* O157:H7 (Table 2). As observed with *Salmonella* counts on treated seeds, *E. coli* O157:H7 counts from both control and scarified seeds receiving the same

TABLE 3. *Populations of* Salmonella *recovered from control, scarified, and polished alfalfa seeds (supplier 2) following chemical treatment*

Seed condition	Inoculum carrier	Treatment	Population (log_{10} CFU/g)[a]		Reduction (log_{10} CFU/g)[d]	Germination (%)[a]
			BSAN[b]	TSAN[c]		
Control	Water	Water (control)	3.95 A	4.15 B		80 LM
		Chlorine (20,000 ppm)	1.30 D–F	1.69 E	2.46	84 J–L
		H_2O_2 (8%)	0.82 F–I	0.46 I	3.69	80 LM
		$Ca(OH)_2$ (1%)	0.10 JK	0.60 I	3.55	80 LM
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	<0.30 K	0.10 I	4.05	80 LM
		$Ca(OH)_2$ (1%) + Span 20 (1%)	0.42 I–K	0.98 I	3.17	81 LM
	HSA (5%)	Water (control)	4.04 A	4.17 B		84 J–L
		Chlorine (20,000 ppm)	1.69 CD	2.02 D	2.15	81 LM
		H_2O_2 (8%)	0.52 H–K	0.43 I	3.75	80 M
		$Ca(OH)_2$ (1%)	0.98 E–H	1.25 I	2.92	89 H–J
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	0.36 I–K	0.66 H	3.51	81 LM
		$Ca(OH)_2$ (1%) + Span 20 (1%)	<0.30 K	0.30 I	3.87	70 N
Scarified	Water	Water (control)	4.35 A	4.45 A		92 B–I
		Chlorine (20,000 ppm)	2.05 BC	2.41 C	2.04	95 A–G
		H_2O_2 (8%)	1.24 D–G	1.68 I	2.77	97 A–D
		$Ca(OH)_2$ (1%)	0.84 F–I	1.23 I	3.22	97 A–D
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	1.04 E–H	1.41 I	3.04	96 A–D
		$Ca(OH)_2$ (1%) + Span 20 (1%)	0.40 I–K	0.80 I	3.65	81 LM
	HSA (5%)	Water (control)	4.45 A	4.53 A		98 A
		Chlorine (20,000 ppm)	2.53 B	2.41 C	2.12	92 C–I
		H_2O_2 (8%)	0.74 G–I	0.52 I	4.01	98 AB
		$Ca(OH)_2$ (1%)	1.23 D–G	1.25 I	3.28	96 A–E
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	1.22 D–F	1.15 F	3.38	97 A–C
		$Ca(OH)_2$ (1%) + Span 20 (1%)	0.30 I–K	0.47 G	4.06	79 LM
Polished	Water	Water (control)	4.16 A	4.22 A		91 D–I
		Chlorine (20,000 ppm)	1.46 DE	1.86 F	2.36	96 A–F
		H_2O_2 (8%)	<0.30 K	<0.30 I[e]	>3.92	90 G–I
		$Ca(OH)_2$ (1%)	0.33 I–K	<0.30 I[e]	>3.92	90 E–I
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	<0.30 K	0.20 HI	4.02	87 I–K
		$Ca(OH)_2$ (1%) + Span 20 (1%)	0.53 H–K	1.01 I	3.21	83 K–M
	HSA (5%)	Water (control)	4.33 A	4.42 A		98 A–C
		Chlorine (20,000 ppm)	1.65 CD	2.16 E	2.26	96 A–D
		H_2O_2 (8%)	<0.30 F	<0.30 I[e]	>4.12	95 A–H
		$Ca(OH)_2$ (1%)	0.58 H-J	0.58 HI	3.84	93 A–H
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	0.79 F–I	1.54 I	2.88	90 F–I
		$Ca(OH)_2$ (1%) + Span 20 (1%)	0.82 F–I	1.31 I	3.11	92 B–I

[a] Values in the same column that are followed by the same letter are not significantly different ($\alpha = 0.05$).
[b] Populations of *Salmonella* recovered on BSA supplemented with 50 μg/ml nalidixic acid.
[c] Populations of *Salmonella* recovered on TSA supplemented with 50 μg/ml nalidixic acid.
[d] Within the same seed condition and inoculum carrier, log_{10} reduction compared with number recovered on TSAN from seeds treated with water (control).
[e] *Salmonella* was detected by enrichment.

inoculum carrier and chemical treatment are not significantly different. The overall trends in reduction of *Salmonella* and *E. coli* O157:H7 populations, however, would suggest that chemical treatments are less efficacious in removing or killing these pathogens on scarified seeds than on nonscarified seeds obtained from supplier 1.

Seeds from supplier 2. Control, scarified, and polished alfalfa seeds obtained from supplier 2 were subjected to the same inoculation and chemical treatments applied to seeds from supplier 1. Results from seeds obtained from supplier 2 that we inoculated with *Salmonella* are shown in Table 3. Although the same water and HSA carriers were used to prepare inocula for control, scarified, and polished seeds, a comparison of numbers of *Salmonella* recovered on TSAN from seeds treated with water (control) reveals that significantly higher ($\alpha = 0.05$) populations were recovered from scarified and polished seeds than from control seeds. This may indicate that more *Salmonella* cells were able to attach or survive on scarified and polished seeds compared to control seeds or that cells were more easily removed from scarified and polished seeds. Recovery of *Salmonella* from scarified and polished seeds treated with water was not significantly influenced by the composition of the carrier. Chemical treatments significantly reduced populations of

TABLE 4. *Populations of* E. coli *O157:H7 recovered from control, scarified, and polished alfalfa seeds (supplier 2) following chemical treatment*

Seed condition	Inoculum carrier	Treatment	Population (log_{10} CFU/g)[a] BSAN[b]	Population (log_{10} CFU/g)[a] TSAN[c]	Reduction (log_{10} CFU/g)[d]	Germination (%)[a]
Control	Water	Water (control)	3.47 B	3.86 A		83 D–H
		Chlorine (20,000 ppm)	1.26 D	1.85 D	2.01	71 JK
		H_2O_2 (8%)	<0.30 G	0.26 KL	3.60	76 IJ
		$Ca(OH)_2$ (1%)	<0.30 G	0.80 F–J	3.06	77 G–J
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	<0.30 G	0.68 F–K	3.18	81 F–I
		$Ca(OH)_2$ (1%) + Span 20 (1%)	0.30 G	0.20 KL	3.66	79 G–I
	HSA (5%)	Water (control)	3.45 B	3.89 A		80 G–I
		Chlorine (20,000 ppm)	1.48 D	1.90 D	1.99	63 L
		H_2O_2 (8%)	<0.30 G	0.20 KL	3.69	66 KL
		$Ca(OH)_2$ (1%)	<0.30 G	0.10 L	3.79	77 H–J
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	0.30 FG	0.85 F–I	3.04	81 F–I
		$Ca(OH)_2$ (1%) + Span 20 (1%)	0.30 G	0.30 J–L	3.59	80 G–I
Scarified	Water	Water (control)	3.68 AB	4.05 A		96 A–C
		Chlorine (20,000 ppm)	1.80 C	2.39 BC	1.66	81 F–I
		H_2O_2 (8%)	<0.30 G	1.75 D	2.30	84 D–H
		$Ca(OH)_2$ (1%)	<0.30 G	0.30 J–L	3.75	97 AB
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	<0.30 G	0.36 I–L	3.69	94 A–C
		$Ca(OH)_2$ (1%) + Span 20 (1%0	0.10 G	0.26 KL	3.79	96 A–C
	HSA (5%)	Water (control)	3.78 AB	4.31 A		95 A–C
		Chlorine (20,000 ppm)	1.90 C	2.50 B	1.81	82 E–I
		H_2O_2 (1%)	<0.30 G	1.80 D	2.51	80 GH
		$Ca(OH)_2$ (1%)	<0.30 G	0.40 H–L	3.91	97 A
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	0.96 E	1.77 D	2.54	96 A–C
		$Ca(OH)_2$ (1%) + Span 20 (1%)	0.50 G	1.12 EF	3.19	97 A
Polished	Water	Water (control)	3.68 AB	4.06 A		94 A–C
		Chlorine (20,000 ppm)	0.86 E	1.51 DE	2.55	82 E–I
		H_2O_2 (8%)	<0.30 G	0.50 G–L	3.56	89 B–E
		$Ca(OH)_2$ (1%)	<0.30 G	0.10 L	3.96	95 A–C
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	0.10 G	0.62 F–L	3.44	90 A–D
		$Ca(OH)_2$ (1%) + Span 20 (1%)	<0.30 G	0.58 G–L	3.48	95 A–C
	HSA (5%)	Water (control)	3.79 A	4.11 A		92 A–C
		Chlorine (20,000 ppm)	1.29 D	1.96 C–D	2.15	81 F–I
		H_2O_2 (8%)	<0.30 G	0.89 F–H	3.22	85 D–G
		$Ca(OH)_2$ (1%)	0.10 G	0.45 G–L	3.66	94 A–C
		$Ca(OH)_2$ (1%) + Tween 80 (1%)	<0.30 G	0.26 KL	3.85	88 C–F
		$Ca(OH)_2$ (1%) + Span 20 (1%)	0.10 G	0.94 FG	3.17	97 A

[a] Values in the same column that are followed by the same letter are not significantly different ($\alpha = 0.05$).
[b] Populations of *E. coli* O157 : H7 recovered on SMA supplemented with 50 μg/ml nalidixic acid.
[c] Populations of *E. coli* O157 : H7 recovered on TSA supplemented with 50 μg/ml nalidixic acid.
[d] Within the same seed condition and inoculum carrier, log_{10} reduction compared with number recovered on TSAN from seeds treated with water (control).

Salmonella by 2.12 to 4.12 log_{10} CFU/g of seeds, a range slightly less than that observed using seeds from supplier 1. Overall, treatment with 20,000 ppm chlorine was less effective than other chemical treatments in eliminating *Salmonella.* Unlike observations from experiments using seeds from supplier 1, a case cannot be made that *Salmonella* is more difficult to remove from scarified seeds than from nonscarified seeds. Likewise, log_{10} reductions in populations on polished seeds subjected to the same inoculation and chemical treatment conditions were not consistently higher or lower than those observed for control seeds. The germination percentage of seeds was significantly increased as a result of scarifying or polishing, indicating that a portion of the untreated seeds exhibited the hard-to-germinate characteristic.

Results from experiments in which seeds from supplier 2 were inoculated with *E. coli* O157:H7 are shown in Table 4. Unlike observations with *Salmonella,* regardless of inoculum carrier, although more *E. coli* were recovered on TSAN from scarified and polished seeds treated with water (control) compared to the number recovered from seeds that were not scarified or polished, the differences were not significant ($\alpha = 0.05$). Chlorine (20,000 ppm) was, overall, less effective than other chemical treatments in eliminating *E. coli* O157:H7, regardless of seed condition or inoculum carrier. Log_{10} reductions in *E. coli* O157:H7 resulting from

a given chemical treatment of scarified or polished seeds were, as observed in experiments using seeds form supplier 2 inoculated with *Salmonella,* not consistently higher or lower than reductions on control seeds. The enhanced germination percentage resulting from scarification or polishing seeds from supplier 2, using *Salmonella* as an inoculum, was confirmed in experiments in which subsamples of the same seeds were inoculated with *E. coli* O157:H7.

In summary, observations using alfalfa seeds from supplier 1 support the hypothesis that scarification exacerbates the problem of removing or killing *Salmonella* and *E. coli* O157:H7. However, observations using scarified or polished seeds from supplier 2 do not provide evidence for this phenomenon. Factors other than these mechanical abrasion treatments also apparently influence the efficacy of chemical sanitizer treatments. Studies using several additional lots of control and mechanically treated seeds need to be done before conclusions can be drawn concerning the impact of mechanical abrasion on the efficacy of chemical treatments to eliminate pathogenic bacteria on alfalfa seeds.

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