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Inactivation of *Escherichia coli* O157:H7 and *Salmonella* on Mung Beans, Alfalfa, and Other Seed Types Destined for Sprout Production by Using an Oxychloro-Based Sanitizer

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ABSTRACT

The efficacy of a stabilized oxychloro-based food grade sanitizer to decontaminate seeds destined for sprout production has been evaluated. By using mung bean seeds as a model system, it was demonstrated that the sanitizer could be used to inactivate a five-strain cocktail of *Escherichia coli* O157:H7 or *Salmonella* introduced onto beans at 10^3 to 10^4 CFU/g. *Salmonella* was more tolerant to stabilized oxychloro than was *E. coli* O157:H7, with sanitizer levels of >150 and >50 ppm, respectively, being required to ensure pathogen-free sprouts. The decontamination efficacy was also found to be dependent on treatment time (>8 h optimal) and the seed-to-sanitizer ratio (>1:4 optimal). Stabilized oxychloro treatment did not exhibit phytotoxic effects, as germination and sprout yields were not significantly ($P > 0.05$) different as compared with untreated controls. Although human pathogens could be effectively eliminated from mung beans, the aerobic plate count of native microflora on sprouts grown from treated seed was not significantly ($P > 0.05$) different from the controls. The diversity of microbial populations (determined through 16S rRNA denaturing gradient gel electrophoresis analysis) associated with bean sprouts was not significantly affected by the sanitizer treatment. However, it was noted that *Klebsiella* and *Herbasprillum* (both common plant endophytes) were absent in sprouts derived from decontaminated seed but were present in control sprouts. When a further range of seed types was evaluated, it was found that alfalfa, cress, flax, and soybean could be decontaminated with the stabilized oxychloro sanitizer. However, the decontamination efficacy with other seed types was less consistent. It appears that the rate of seed germination and putative activity of sanitizer sequestering system(s), in addition to other factors, may limit the efficacy of the decontamination method.

Sprouted seeds have a high nutritive value, in addition to anticholesterol and anticarcinogenic constituents *(11)*. However, sprouted seeds continue to be implicated in outbreaks of foodborne illness and hence are considered a significant food safety risk *(16)*. One of the largest outbreaks recorded occurred within Ontario, Canada, in 2005. Here over 600 reported cases of salmonellosis were traced to contaminated mung bean sprouts *(7)*. To date, the majority of cases have been linked to alfalfa and bean sprouts *(15)*, although other seed types have been identified as potential vehicles for human pathogens *(4, 5, 32)*. *Salmonella* is the most frequently isolated human pathogen on sprouts, although cases involving *Escherichia coli* O157:H7 have also been reported *(2, 30, 36, 37)*.

In the majority of foodborne illness outbreaks, the seed used for sprout production has been shown to be the most significant source of human pathogens *(2)*. Therefore, the U.S. Food and Drug Administration issued guidelines recommending that all seed destined for sprout production should be decontaminated with calcium hypochlorite treatment (20,000 ppm for at least 15 min) *(2, 14, 39)*. Although seed decontamination has improved the safety of sprouted seeds, a number of sporadic outbreaks of foodborne illness and product recalls associated with sprouts continue to occur *(16)*.

The limitation of calcium hypochlorite has led to a sustained effort to find alternative seed decontamination methods. However, the majority of interventions (chemical and physical) evaluated to date can reduce, but not eliminate, pathogens on seeds *(15, 21, 42)*. Yet, successful mung bean decontamination has been achieved by applying acetic acid vapor for 12 h at 45°C *(10)*. Mung beans held at 55°C for 4 to 7 days has also been reported to inactivate pathogens inoculated onto mung beans *(24)*. Dry heat (50°C for 1 h) in combination with irradiation (2.5 kGy) has been shown to eliminate *E. coli* O157:H7 on alfalfa, mung bean, and radish seeds, with negligible impact on germination and yield *(3)*. However, given the need to heat seeds, together with the expense of irradiation, the actual feasibility of applying the method in commercial practice is questionable.

The difficulty encountered with decontaminating seeds can be based on multiple factors. For example, human pathogens can be located within shielded sites on seed coats (crevices, damaged areas), thereby being protected from antimicrobial treatments *(9)*. Any residual human pathogens (even at levels <0.1 CFU/g) surviving the seed decontamination treatment can grow to high levels during the sub-

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TABLE 1. Escherichia coli *O157:H7 and* Salmonella *strains/serovars used in the study*

Escherichia coli O157:H7 strain	Source	*Salmonella enterica* serovar	Source
E. coli O157:H7-C1033[a]	Water sediment	Meleagridis E1[b]	Alfalfa sprouts
E. coli O157:H7-C1032[a]	Soil	Oranienburg C1[b]	Alfalfa sprouts
E. coli O157:H7-C652[a]	Clinical	Newport C2[b]	Alfalfa sprouts
E. coli O157:H7-C476[a]	Clinical	Senftenburg[b]	Alfalfa sprouts
E. coli O157:H7-C477[a]	Clinical	Montevideo[a]	Tomatoes

[a] Strains obtained from Canadian Research Institute for Food Safety, University of Guelph, Guelph, Ontario, Canada.
[b] Strains donated by Dr. C. Poppe, Health Canada, Guelph, Ontario, Canada.

sequent sprouting process *(22).* Therefore, seed decontamination treatments applied must ensure complete inactivation of human pathogens while at the same time maintaining the viability and vigor of the seed.

In the following study, a stabilized oxychloro (SOC)-based sanitizer (commercially known as Germin-8-or) to decontaminated seeds has been evaluated. The sanitizer is essentially composed of a stabilizing agent and traces of chlorate, with chlorite constituting the primary antimicrobial agent. Chlorite is typically used within the food industry in its acidic form to generate chlorine dioxide *(19).* In its nonacidified form, chlorite is not considered a suitable biocide because of its relative instability (typically stable in water at neutral pH for 48 h) and low antimicrobial activity over short contact times *(20, 25, 26).* However, over extended contact times (>6 h), chlorite has been shown to exhibit bactericidal properties without causing significant cytotoxic affects against mammalian cells or fungi *(25).*

In the course of sprout production, it is standard practice to soak seeds for 3 to 16 h to stimulate the germination process *(2).* Therefore, by the inclusion of SOC at the seed soak stage, it is possible to expose human pathogens to the bactericidal agent over long periods, without any adverse affects on sprout development. The objectives of the current study were to optimize and determine the efficacy of SOC treatment to inactivate human pathogens on a range of different seed types used in sprout production.

MATERIALS AND METHODS

Bacterial strains and preparation of inocula. *E. coli* O157:H7 and *Salmonella* used in the study were composed of environmental, clinical, tomato, or sprout isolates (Table 1). An aliquot (1 ml) of an overnight culture of the individual *E. coli* O157:H7 or *Salmonella* strains was transferred into 50 ml of tryptic soy broth (Difco, Becton Dickson, Sparks, Md.) and incubated for 24 h at 37°C. Bacterial cells were harvested by centrifugation (5,500 × *g* for 10 min at 4°C) and washed once in 0.8% saline. The final cell pellet was resuspended in saline to a cell density of 10^6 CFU/ml (ca. OD_{600} of 0.2). Equal volumes of the five different *E. coli* O157:H7 or *Salmonella* suspensions were then combined to produce a cocktail that was subsequently used to inoculate seeds.

Inoculation of seeds. Mung bean, alfalfa, broccoli, buckwheat, clover, chickpeas, cress, flax, mustard, onion, radish, soybean, sesame, and sunflower seeds were obtained from Mumms Seeds, Ltd. (Parkside, Saskatchewan, Canada). At least two separate batches of the different seed types were used during the course of the study.

Seeds (250 g) were soaked in 250 ml of the five-strain cocktail of *E. coli* O157:H7 or *Salmonella* for 20 min. The seeds were then transferred to sterile filter paper within a biological safety cabinet and allowed to dry at ambient temperature for 48 to 60 h. The inoculated seeds were then used immediately or stored at 4°C until required (maximum of 5 days).

Seed decontamination and sprouting. Contaminated seeds (25 g) were soaked for defined periods in 250 ml of the appropriate concentration (25 to 200 ppm) of SOC solution (Vernagene, Ltd., Bolton, Lancashire, UK) at 28°C. The seeds were then removed, rinsed with distilled water, and germinated for up to 4 days at 28°C in 1.5-liter plastic containers placed in an environmental chamber (Percival Scientific, Perry, Iowa). The sprouting seeds were watered daily with a 5-min soak in 500 ml of sterile distilled water. In parallel, control inoculated mung bean batches were soaked for 20 min in 20,000 ppm calcium hypochlorite (Fisher, Ottawa, Ontario, Canada) prepared in phosphate buffer (50 mM, pH 6). Verification of the free chlorine concentration was performed with a commercially available chlorine test kit (Fisher). The beans were rinsed with distilled water and sprouted as described above.

Microbiological analysis. Microbiological analysis was performed on both the inoculated seeds and sprouts. Duplicate samples (1 g) of inoculated seed were suspended in 9 ml of buffered peptone water (0.1%; Oxoid, Basingstoke, UK) and vortexed for 1 min. For sprouts, duplicate 25-g samples were suspended in 225 ml of buffered peptone water and stomached for 90 s at 230 rpm (model 400, A. J. Steward and Co., London, UK). Dilution series were prepared from the seed wash and sprout homogenates in saline prior to plating onto the appropriate agar medium. Aerobic plate counts were enumerated on tryptic soy agar (Oxoid) incubated at 30°C for 48 h. *Salmonella* was enumerated on either xylose lysine deoxychlorate (Oxoid) or brilliant green (Oxoid) agar incubated at 42°C for 24 h. *E. coli* O157:H7 was enumerated on sorbitol MacConkey agar containing cefixime and tellurite (Difco, Becton Dickinson) incubated at 37°C for 24 h.

Sprout samples (25 g) were tested for the presence of *Salmonella* with the method described in the Canadian Compendium of Analytical Methods, MFHPB 20 *(6).* Sprouts were suspended in 225 ml of buffered peptone water and incubated at 42°C for 24 h. An aliquot (0.1 ml) of the enriched culture was then inoculated into the center of a semisolid Rappaport-Vassiliadis plate (Oxoid) that was subsequently incubated at 37°C for 24 h. Cells from the outer perimeter of the growth halo (presumptive motile *Salmonella*) were streaked onto brilliant green agar (Oxoid) and incubated at 37°C overnight. The Oxoid *Salmonella* Latex Test FT0203 was used for serological confirmation of typical colonies (i.e., red colonies surrounded by brilliant red zones).

Sprouts were enriched for *E. coli* O157:H7 with buffered

peptone water containing 0.5% (wt/vol) sodium thioglycolate incubated at 37°C for 24 h *(36).* Aliquots (10 μl) of the enriched culture were then streaked onto cefixime and tellurite agar that was subsequently incubated at 37°C for 24 h. Typical colonies (colorless) were confirmed as *E. coli* O157 with the Oxoid *E. coli* O157 Latex Test DR0620M.

DNA typing of *E. coli* O157:H7 and *Salmonella*. Enterobacterial repetitive intergenic consensus (ERIC)–PCR *(40)* was used to identify which of the five strains of *E. coli* O157:H7 or serovars of *Salmonella* were present on sprouts upon completion of the sprouting process. Up to five colonies from the positive selective agar plates were picked onto Luria-Bertani agar (Difco, Becton Dickinson) that was subsequently incubated at 37°C for 24 h. Single colonies were then suspended in 0.2 ml of Tris-EDTA buffer heated at 100°C to lyse cells. Cell debris was removed by centrifugation (13,000 × *g*) and the supernatant, containing DNA, decanted into a new Eppendorf tube.

The PCR reactions were carried out with a final volume of 25 μl, containing 24 μl of master mix and 1 μl of the bacterial cell supernatant (DNA template). In all, the primers used were ERIC 1 (forward) 5′-ATGTAAGCTCCTGGGGATTCAC-3′ and ERIC 2 (reverse) 5′-AAGTAAGTGACTGGGGTGAGCG-3′. The final PCR reaction contained 100 pM of each primer, 1 U of *Taq* DNA polymerase (New England BioLabs, Inc., Ipswich, Mass.), 0.2 mM each of the deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, and dTTP; Sigma, St. Louis, Mo.), 2.0 mM $MgCl_2$ (Promega, Madison, Wis.), and Buffer III (1× concentration; Boehringer Mannheim, Ltd., Burlington, Ontario, Canada). PCR was performed in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, Calif.). Amplification parameters included 1 cycle at 94°C for 3 min, and 35 cycles at 94°C for 30 s, 36°C for 90 s, and 72°C for 4 min. The final cycle was 8 min at 72°C. PCR products were separated on 2% (wt/vol) agarose gels (Fisher) prepared in Tris-borate-EDTA buffer supplemented with ethidium bromide (0.5 μg/ml). All amplified DNA fragments were separated at 75 V for 2.5 h with a 100-bp DNA ladder (Sigma) acting as a molecular weight marker. After electrophoresis the gels were visualized and digital images captured with an image analyzer (Bio-Rad Laboratories, Mississauga, Ontario, Canada). DNA patterns were analyzed, and dice similarity coefficients calculated with Molecular Analyst Software, Bio-Rad Fingerprinting II, version 3.0 (Bio-Rad Laboratories, Hercules, Calif.).

DGGE. Denaturing gradient gel electrophoresis (DGGE) of native microflora was performed to determine if decontaminating seeds with SOC affected the native sprout microbiota. Noninoculated mung beans were soaked in 200 ppm SOC for 24 h at 28°C. The beans were then rinsed with distilled water and sprouted for a further 3 days with periodic watering. Sprouts samples (10 g) were collected from each batch and suspended in 90 ml of buffered peptone water (0.1%) prior to stomaching. DNA was extracted directly from the sprout homogenates with a commercial kit (DNeasy, Qiagen, Inc., Mississauga, Ontario, Canada) according to the manufacturer's instructions.

For the PCR-based DGGE analysis, a fragment of the ribosomal 16S rRNA gene containing the V2–V3 region (position 339 to 539 in *E. coli* gene) was targeted. This region was amplified from genomic DNA of the sprout microflora using primers HDA1-GC (5′-**CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G**AC TCC TAC GGG AGG CAG CAG T-3′; the GC clamp is in boldface) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′) as previously described *(41).* PCR amplification was carried out in a total volume of 50 μl containing reaction buffer (10 mM Tris-HCl, pH 8.3, containing 2.5 mM $MgCl_2$ and 50 mM KCl), 0.2 mM of each deoxynucleoside triphosphate, 20 pmol of each primer, 500 ng of bacterial DNA, and 2.5 U of *Taq* DNA polymerase. The amplification program was 94°C for 4 min; 30 cycles of 94°C for 30 s, 56°C for 30 s, and 68°C for 60 s; and finally, 68°C for 7 min.

Amplicons were separated by DGGE with the Bio-Rad DCode system (Bio-Rad Laboratories). The polyacrylamide gels consisted of 10% polyacrylamide (ratio of acrylamide to bisacrylamide, 37.5:1) and contained a 35 to 65% gradient of urea and formamide, which increased in the direction of electrophoresis. A 100% denaturing solution contained 40% (vol/vol) formamide and 7 M urea. Electrophoresis was performed for 16 h at 70 V in Tris-acetate-EDTA buffer (pH 8.0) at a constant temperature of 60°C. The gels were then silver stained until sufficient band intensity had been attained.

Bands of interest were cut from the gel, and DNA was extracted using QIAquick Gel Extraction Kit (Qiagen). DNA samples were amplified using PCR and sequenced by Laboratory Services (University of Guelph, Guelph, Ontario, Canada). The obtained sequences were cross-matched with those contained within the GenBank, European Molecular Biology Laboratory and DNA Data Bank of Japan databases by using the BLAST algorithm.

SOC and protein concentration in spent soak water. SOC was measured as $NaOCl_2$ with the method described by Ingram et al. *(26).* Seeds were soaked in 200 ppm SOC solution for 24 h as previously described. Aliquots (50 μl) of spent soak water were added to 50 μl of 2% (wt/vol) KI and 1.5 ml of 50 mM HCl, with the formation of iodine being detected spectrophotometrically at 350 nm. Residual chlorite in sprouts derived from seeds treated with SOC was also tested. Batches of sprouts (10 g) were suspended in 90 ml of distilled water and homogenized. The extract was centrifuged (8,000 × *g* for 20 min) to remove sprout debris and chlorite within the supernatant quantified by using the assay described above. Calibration curves were prepared with $NaOCl_2$ solutions ranging from 1 to 200 ppm.

The protein content of spent soak water samples (5 ml) was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories) according to the manufacturer's instructions.

Statistical analysis. All experiments were performed at least three times with duplicate samples being tested on each occasion. Enumeration data was transformed into log values prior to analysis by analysis of variance and Tukey's test (S-Plus, Insightful Corp., New York). In all cases the significance level was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Optimization of mung bean decontamination treatment. SOC treatment did not significantly ($P > 0.05$) affect the extent of germination (>97%), sprout yield, or appearance when applied within the range of 0 to 200 ppm, compared with nontreated controls (Table 2). However, at >2,000 ppm SOC, there was a decrease in seed germination to <25%, and sprout development was stunted.

The aerobic plate counts recovered from mung beans were high but comparable to values reported by others *(33).* *E. coli* O157:H7 was relatively sensitive to SOC with seed treatment using 100 ppm, resulting in pathogen-free sprouts (Table 3). In contrast, a higher level of 200 ppm SOC was required to inactivate *Salmonella* (Table 3). Because of the

TABLE 2. *Bean sprout yield from mung beans decontaminated with SOC sanitizer*

SOC (ppm)	Bean sprout yield (g)[a]
0	421 ± 12 A
25	444 ± 28 A
100	447 ± 8 A
125	408 ± 3 A
150	419 ± 20 A
175	421 ± 15 A
200	426 ± 23 A

[a] Mung bean batches (10 g) were soaked for 24 h at 28°C in distilled water (control) or SOC solution, and the weight of sprouts determined following a 4-day sprouting period. Means followed by the same letter are not significantly ($P > 0.05$) different.

higher tolerance of *Salmonella* to SOC, a concentration of 200 ppm was selected for subsequent studies.

Treatment of seeds with 20,000 ppm free chlorine from calcium hypochlorite did not lead to the elimination of the two pathogens from mung beans, with high levels of both *Salmonella* and *E. coli* O157:H7 being detected in sprouts grown from the treated seed (Table 3). This result is in agreement with the studies performed by Fett *(14),* who also reported that calcium hypochlorite applied at the recommended levels was insufficient to eliminate pathogens introduced onto mung beans. Therefore, the results confirm that SOC is a more effective sanitizer for decontaminating seeds as compared with calcium hypochlorite.

Through DNA fingerprinting, *Salmonella enterica* serovar Meleagridis was the only serovar recovered from sprouts derived from mung beans treated with an ineffective concentration of SOC (150 ppm; Table 3). The dominance of Meleagridis was not associated with the inability of the other *Salmonella* to grow on sprouting seeds, as individually inoculated serovars attained comparable counts on bean sprouts at day 4 (results not shown). Howard and Hutcheson *(23)* also reported that the growth of *Salmonella* on sprouting alfalfa seed was serovar independent. However, the same workers did note that a *Salmonella* Cubana strain, originally isolated from alfalfa sprouts, had a higher growth rate as compared with isolates derived from meat or clinical sources *(23).* The authors concluded that, although different serovars of *Salmonella* can grow on sprouting seeds, the actual growth rates vary, which ultimately affects which strain becomes dominant on alfalfa sprouts. This may be the underlying reason for the dominance of Meleagridis in bean sprouts encountered in the current study. It is also possible that Meleagridis had enhanced tolerance to SOC treatment. This is unlikely, as individual serovars introduced onto beans were equally sensitive to SOC treatment (results not shown).

Corresponding ERIC-PCR DNA fingerprint studies were performed with *E. coli* O157:H7 isolates recovered from bean sprouts derived from beans treated with 25 ppm SOC. However, the banding patterns obtained for the five strains of *E. coli* O157:H7 all exhibited close similarity, making differentiation problematic. Although ERIC-PCR has previously been applied to differentiate *E. coli* O157:H7, the technique has relatively low resolving power, compared with pulsed-field gel electrophoresis or phage typing *(18, 29).* Therefore, in the present study it was not possible to establish which of the *E. coli* O157:H7 introduced on mung beans became dominant on the subsequent bean sprouts. However, the relative sensitivity of *E. coli* O157:H7 to SOC would suggest that no significant intrastrain resistance to the sanitizer exists.

Although *E. coli* O157:H7 and *Salmonella* were effectively inactivated on mung beans by SOC treatment at >50 and >150 ppm, respectively, the aerobic plate counts did not significantly ($P > 0.05$) differ between treated and nontreated sprout batches (Table 3). This would suggest that the endogenous microflora was unaltered by the SOC treatment. Indeed, through 16S rRNA analysis it was demonstrated that the microbial populations associated with bean sprouts derived from SOC-treated seeds (noninoculated) were similar to nontreated controls. However, it was noted that bands corresponding to *Klebsiella* and *Herbasprillum* were missing in sprouts derived from decontaminated beans (Fig. 1). Both bacteria are common endophytes and frequently recovered from plants including sprouted seeds *(12,*

TABLE 3. *Effect of SOC concentration on the efficacy of mung bean decontamination and subsequent aerobic plate and pathogen counts on sprouted seed*[a]

Sanitizer	Concn (ppm)	Log CFU/g sprouts[b]		
		APC[c]	*Salmonella*	*E. coli* O157:H7
Control	0	9.44 ± 0.38 A	9.10 ± 0.26 A	9.12 ± 0.66 A
SOC	50	NT[d]	8.87 ± 0.43 A	9.09 ± 0.60 A
	100	9.52 ± 0.52 A	5.30 ± 0.20 B	ND[e] B
	150	9.25 ± 0.36 A	4.90 ± 0.32 B	ND B
	200	9.46 ± 0.58 A	ND C	ND B
Calcium hypochlorite	20,000	9.45 ± 0.33 A	7.96 ± 0.86 A	8.59 ± 0.40 A

[a] Initial loading on mung beans was 10^3 to 10^4 CFU/g in all cases.
[b] Means within columns followed by the same letter are not significantly ($P > 0.05$) different.
[c] APC, aerobic plate count. Values are from sprouts derived from treated beans inoculated with *E. coli* O157:H7.
[d] NT, not tested.
[e] ND, not detected (<1 CFU/25 g).

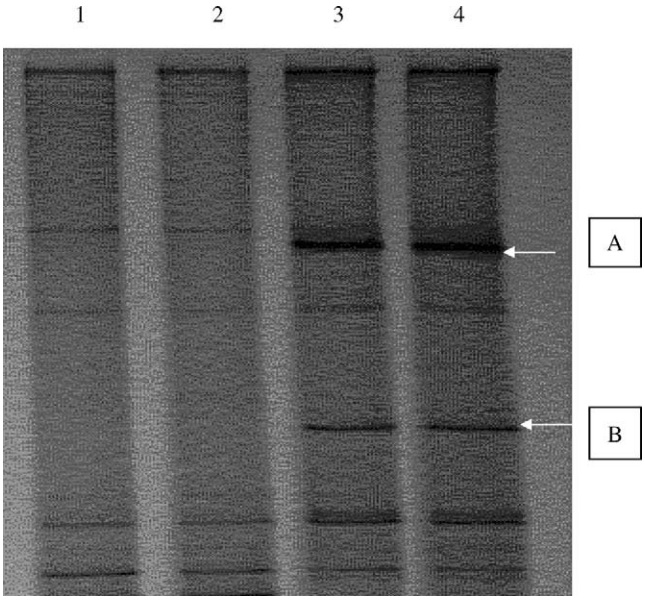

FIGURE 1. *DGGE analysis showing profiles of 16S rRNA amplified from the microbiota of bean sprouts derived from SOC-treated (lanes 1 and 2) and nontreated (lanes 3 and 4) mung beans. Arrowheads correspond to* Klebsiella *(a) and* Herbasprillum *(b).*

13, 34), so their presence was not unexpected. However, it is unclear why the other microbial populations associated with bean sprouts were apparently insensitive to SOC. It could be that a proportion of the populations was present deep within the seed or alternatively, the bacterial populations represent posttreatment contamination derived from the environment and/or irrigation water. From the limited studies on the microbial ecology of sprouts, it is believed that seedborne contamination is of greater significance than the sprouting environment *(28).* However, this may not apply to decontaminated seed, where it can be assumed the endogenous microflora would have been reduced thereby enabling environmental microbiota to become established on the developing sprouts.

It has been previously reported that human pathogens can potentially undergo recovery after sublethal exposure to sanitizers *(24).* In the present study, sprouts testing negative at 48 h did not yield a positive reaction for either *E. coli* O157:H7 or *Salmonella* when additional samples were taken 96 h into the sprouting process. Therefore, no evidence of posttreatment recovery of either pathogen was observed. In this respect, SOC treatment is compatible with the current recommendation to screen for human pathogens in spent irrigation water 48 h into the sprouting process *(2).*

Through further optimization studies, it was found that the minimum mung bean-to-SOC ratio was 1:4 (wt/vol) to achieve consistent inactivation of *E. coli* O157:H7 or *Salmonella* (Table 4). Currently, there are no specific recommendations on the seed-to-sanitizer ratio that should be applied during seed decontamination *(2, 38).* However, the results obtained in this study clearly illustrate that for SOC at least, a defined seed-to-sanitizer ratio should be employed.

The contact time required to ensure elimination of human pathogens on seeds was between 8 and 19 h (Table 5). The reasons for the extended soaking time to ensure the effective decontamination of mung beans are unclear. However, it can be assumed that the sanitizer absorption rates vary among seeds within the same batch. Therefore, a long contact time is required to ensure that all pathogens are released from protective sites on the seed coat and subsequently inactivated. This is especially relevant considering that even low levels of surviving human pathogens can grow to high levels during the sprouting process *(17, 23).*

TABLE 4. *Effect of mung bean to SOC sanitizer ratio on the efficacy of seed decontamination to eliminate* Escherichia coli *O157:H7 or* Salmonella

Bean-to-sanitizer ratio (wt/vol)	Treatment	Sprouts positive by enrichment (positive/no. tested)[a]	
		E. coli O157:H7	*Salmonella*
1:1	Nontreated[b]	4/4	4/4
	Treated[c]	4/4	4/4
1:2	Nontreated	4/4	4/4
	Treated	4/4	4/4
1:4	Nontreated	4/4	4/4
	Treated	0/4	0/4
1:6	Nontreated	4/4	4/4
	Treated	0/4	0/4
1:8	Nontreated	4/4	4/4
	Treated	0/4	0/4
1:10	Nontreated	4/4	4/4
	Treated	0/4	0/4

[a] Inoculated mung beans (25 g) were soaked in SOC solutions at different seed-to-sanitizer ratios. After 24 h the seeds were removed and sprouted for a further 48 h at 28°C. The sprouts were then enriched for either *Escherichia coli* O157:H7 or *Salmonella.*

[b] Nontreated: inoculated mung beans soaked in distilled water for 24 h.

[c] Treated: inoculated mung beans soaked in 200 ppm SOC sanitizer for 24 h.

Decontamination of different seed types. The efficacy of the optimized SOC treatment to decontaminate a broad range of seed types was evaluated. It was noted that

TABLE 5. *Effect of SOC sanitizer (200 ppm) contact time on mung bean decontamination efficacy (*n = *4)*

Contact time (h)	No. of positive sprouts by enrichment/no. tested[a]	
	E. coli O157:H7	*Salmonella*
4	4/4	4/4
8	4/4	4/4
19	0/4	0/4
24	0/4	0/4

[a] Inoculated mung beans (25 g) were soaked in SOC solutions for different periods. The seeds were removed and sprouted for a further 48 h at 28°C prior to enrichment for either *Escherichia coli* O157:H7 or *Salmonella.*

TABLE 6. *Decontamination of a range of seed types inoculated with* Salmonella *or* Escherichia coli *O157:H7 with SOC sanitizer*

Seed type	Treatment	No. of sprouts positive by enrichment/no. tested	
		E. coli O157:H7	*Salmonella*
Soybeans	Nontreated[a]	4/4	4/4
	Treated (400 ppm)[b]	0/4	0/4
Alfalfa	Nontreated	4/4	4/4
	Treated (200 ppm)	0/4	0/4
Cress	Nontreated	4/4	4/4
	Treated (200 ppm)	0/4	0/4
Flax	Nontreated	4/4	4/4
	Treated (200 ppm)	0/4	0/4
Clover	Nontreated	4/4	4/4
	Treated (200 ppm)	0/4	2/4
Mustard	Nontreated	4/4	4/4
	Treated (400 ppm)	2/4	1/4
Radish	Nontreated	3/4	3/4
	Treated (200 ppm)	2/4	3/4
Chick pea	Nontreated	4/4	4/4
	Treated (200 ppm)	4/4	4/4
Broccoli	Nontreated	4/4	4/4
	Treated (200 ppm)	4/4	4/4
Sunflower (dehulled)	Nontreated	3/3	3/3
	Treated (400 ppm)	3/3	3/3
Sunflower (with hulls)	Nontreated	3/3	3/3
	Treated (400 ppm)	3/3	3/3
Buckwheat	Nontreated	3/3	3/3
	Treated (400 ppm)	3/3	3/3
Sesame	Nontreated	3/3	3/3
	Treated (400 ppm)	3/3	3/3
Onion	Nontreated	3/3	3/3
	Treated (400 ppm)	3/3	3/3

[a] Nontreated: inoculated seeds soaked in distilled water for 24 h.
[b] Treated: inoculated seeds soaked in 200 or 400 ppm SOC sanitizer for 24 h.

with all the seed types tested, both *Salmonella* and *E. coli* O157:H7 became established on the subsequent sprouts (Table 6). This would suggest that although the majority of foodborne illness outbreaks are associated with alfalfa and mung bean, most, if not all, sprouted seed could potential act as vehicles for human pathogens.

Alfalfa, soybean, flax, and cress seed inoculated with either a five-strain or five-serovar cocktail of *E. coli* O157:H7 or *Salmonella* could be consistently decontaminated (Table 6). However, with clover, mustard, and radish, variable results were obtained. Although increasing the SOC concentration to 400 ppm enhanced the decontaminating efficacy for mustard without decreasing sprout yield, this was found to significantly decrease the yield with clover, radish, broccoli, and chickpea. SOC treatment of other seed types was unsuccessful even when applied at 400 ppm over longer (48 h) contact times (results not shown).

The effect of seed type on the efficacy of SOC treatment was unexpected. However, it was noted that those seeds that were not successfully decontaminated with the sanitizer germinated at a slower rate as compared with mung beans or alfalfa. Therefore, it is possible that the protective sites within the seed shielded human pathogens from the antimicrobial action of the sanitizer. An additional possibility is that the SOC acted synergistically with exudates released by the seed to inactivate pathogens. Indeed, the extracts from several seed types have found to be potent antimicrobial agents *(1, 8, 31).* Therefore, it is possible the antimicrobials released by seeds such as mung beans were higher as compared with those seeds that could not be decontaminated. This hypothesis was tested by treating a mixture of mung beans and radish seed with SOC prior to sprouting (Table 7). However, even a low proportion of radish seed added to a batch of mung beans was sufficient to reduce the efficacy of the SOC decontamination treatment, with *Salmonella* being recovered from the subsequent sprouts. This would suggest that SOC was being sequestered by germinating radish seeds as opposed to an antimicrobial being released by mung beans. It has been reported that chlorite can be readily sequestered by protein *(27).* However, when the protein content in spent soak solutions was assayed, content derived from mung bean (132 μg/ml) was significantly ($P < 0.05$) higher when compared with either radish (57 μg/ml) or soybean (37 μg/ml). Therefore, protein content cannot explain the low efficacy of SOC to decontaminate the different seed types.

TABLE 7. *Efficacy of SOC sanitizer in decontaminating* Salmonella-*inoculated radish seed and mung bean mixtures*

Ratio of mung beans to radish seeds[a]	No. of sprouts positive by enrichment/no. tested
100% mung bean	0/4
1:1	4/4
2:1	4/4
1:2	4/4
100% radish	4/4

[a] Mung beans were mixed with varying quantities of radish seeds and soaked in SOC (200 ppm) for 24 h at 28°C. The seed mixtures were then sprouted for 48 h with daily watering, and sprouts were screened for the presence of *Salmonella.*

When the residual SOC present in the spent soak solution was determined, it was noteworthy that the levels in samples taken from mung beans or soybean (72 to 74 ppm) soak water were significantly ($P < 0.05$) higher as compared with radish (32 ppm) or broccoli (28 ppm). This may indicate that with the latter seed types, the sanitizer concentration was reduced to below biocidal levels during the treatment period. However, it was noted that the residual level of spent soak water from alfalfa seeds after the 24 h treatment period was only 7 ppm despite the subsequent sprouts testing negative for *Salmonella.* This apparent discrepancy can be explained by the relatively rapid inactivation of human pathogens on alfalfa. Here the inactivation of *Salmonella* on alfalfa could be achieved in <8 h, compared with 8 to 19 h found with mung beans (results not shown). Therefore, it can be proposed that although the levels of active SOC decrease during the course of alfalfa

seed soaking, sufficient sanitizer is present at the critical part of the germinating stage to inactivate human pathogens.

As with all chemical-based sanitizers, there is a risk of chemical residues being associated with the sprouts at the end of the sprouting process. However, in the present case all sprout samples tested did not contain residual SOC (<1 ppm chlorite). This can be attributed to the endogenous SOC-sequestering systems associated with the sprouts and the multiple washing (irrigation) steps applied during sprouting process.

In conclusion, a seed decontamination treatment based on supplementing soak solutions with SOC has been evaluated. Although SOC was ineffective in decontaminating all seed types, several of major commercial importance (e.g., mung bean, alfalfa, and soybean) could be successfully treated. Therefore, SOC represents an alternative to the currently recommended calcium hypochlorite treatment. Future work will evaluate the efficacy of SOC to decontaminate naturally contaminated seed, in addition to evaluating sanitizer performance on a commercial scale.

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