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Combined effects of water activity, temperature and chemical treatments on the survival of *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds

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Aims: The objective of this study was to determine the combined effects of water activity (a_w), chemical treatment and temperature on *Salmonella* and *Escherichia coli* O157:H7 inoculated onto alfalfa seeds.

Methods and Results: Alfalfa seeds inoculated with *Salmonella* or *E. coli* O157:H7 and adjusted to various a_w values were subjected to simultaneous and separate treatments with chemicals and heat. The rate of death of both pathogens was correlated with increased a_w (0·15–0·60) and temperature (5–37°C) over a 52-week storage period. Higher seed a_w enhanced the inactivation of pathogens on seeds heated at 50–70°C for up to 24 h. Treatment of seeds with water, 1% $Ca(OH)_2$, 1% Tween 80, 1% $Ca(OH)_2$ plus 1% Tween 80 or 40 mg l^{-1} Tsunami 200® at 23 or 55°C for 2 min significantly ($\alpha = 0{\cdot}05$) reduced populations of *Salmonella* and *E. coli* O157:H7.

Conclusions: Overall, at the combinations of temperature and concentrations of chemicals tested, 1% $Ca(OH)_2$ was most effective in killing *Salmonella* and *E. coli* O157:H7 without reducing seed viability.

Significance and Impact of the Study: None of the treatments evaluated in this study, whether applied separately or in combination, eliminated *Salmonella* or *E. coli* O157:H7 on alfalfa seeds without sacrificing the viability of the seeds. It remains essential that practices to prevent the contamination of alfalfa seeds be strictly followed in order to minimize the risk of *Salmonella* and *E. coli* O157:H7 infections associated with sprouts produced from these seeds.

INTRODUCTION

Documented outbreaks of *Salmonella* and *Escherichia coli* O157:H7 infections associated with seed sprouts have occurred with increased frequency in the USA during the last decade (National Advisory Committee on Microbiological Criteria for Foods 1999; Taormina *et al*. 1999). The majority of outbreaks have implicated alfalfa sprouts, although infections involving clover and mung bean sprouts have also been documented. Concern about the microbiological safety of seed sprouts is not limited to the USA, however, as outbreaks of salmonellosis associated with mung bean sprouts in the UK (O'Mahony *et al*. 1990), mustard cress sprouts grown in the UK from seeds imported from The Netherlands (Joce *et al*. 1990) and alfalfa sprouts in Finland, Sweden (Ponka *et al*. 1995; Puohiniemi *et al*. 1997), Denmark and Canada (Van Beneden *et al*. 1999) have been reported. Outbreaks involving more than 6000 culture-confirmed cases of *E. coli* O157:H7 infections associated with radish sprouts have occurred in Japan (Gutierrez 1997; Ministry of Health and Welfare of Japan 1997).

The isolation of *Salmonella* or *E. coli* O157:H7 from seeds used to produce sprouts epidemiologically linked to outbreaks of infections is rare. A notable exception was the isolation of *Salmonella* Newport, *Salm.* Albany and *Salm.* Schwarzengrund from alfalfa seeds implicated in a 1996 outbreak of salmonellosis in the USA and Canada (Inami

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and Moler 1999). Harmon *et al*. (1987) detected *Bacillus cereus* in 57% of sprouting seeds examined and Park and Sanders (1990) reported that 40% of the lots of mung bean seeds examined contained *Klebsiella pneumoniae*. Coagulase-positive staphylococci were detected in 12% of sprouting seeds examined by Prokopowich and Blank (1991). Illness traced to the consumption of vegetable sprouts contaminated with *B. cereus* has been described (Portnoy *et al*. 1973) but gastroenteritis caused by *Kl. pneumoniae* and *Staphylococcus aureus* on seed sprouts has yet to be documented.

The reported presence on sprouting seeds of several bacterial species capable of causing illness has raised interest in developing methods for disinfection. Hypochlorites, chlorine dioxide and acidified sodium chlorite are among the chlorine compounds evaluated for their efficacy in killing *Salmonella* and *E. coli* O157:H7 on alfalfa seeds (Beuchat 1997; Beuchat *et al*. 2001; Jaquette *et al*. 1996; Lang *et al*. 2000; Piernas and Guiraud 1997; Taormina and Beuchat 1999; Weissinger and Beuchat 2000). Organic acids (Taormina and Beuchat 1999; Lang *et al*. 2000; Weissinger and Beuchat 2000) and highly alkaline solutions (Beuchat 1997; Beuchat *et al*. 2001; Weissinger and Beuchat 2000) have also been examined for their effectiveness in killing pathogens on alfalfa seeds intended for sprout production. The treatment of contaminated seeds with gaseous acetic acid (Delaquis *et al*. 1999) and potentially lethal volatile compounds from plants (Park *et al*. 2000; Weissinger *et al*. 2001) has been investigated with the assumption that permeation of these volatile compounds to sites where cells of pathogens are lodged can be achieved. To date, none of these treatments has been shown to eliminate human pathogens from seeds intended for sprout production.

The prospect of combining two or more types of treatment, either simultaneously or in sequence, for the purpose of achieving greater reductions in numbers of pathogens on alfalfa seeds has received some research attention. Sequential or combined treatments with lactic acid, hypochlorite and heat (Lang *et al*. 2000) or calcium hydroxide and detergent (Weissinger and Beuchat 2000) have shown promise. The study reported here was undertaken to determine the combined effects of water activity (a_w), temperature and chemical treatment on lethality to *Salmonella* and *E. coli* O157:H7 inoculated onto alfalfa seeds.

MATERIALS AND METHODS

Five serotypes of *Salmonella* were used: Anatum, Cubana, Infantis and Stanley, all from outbreaks of salmonellosis associated with alfalfa sprouts, and Montevideo, from an outbreak associated with raw tomatoes. Five enterohaemorrhagic *E. coli* O157:H7 strains were used: 932 (human isolate), 994 (salami isolate), E0018 (calf faecal isolate), H1730 (human isolate from an outbreak associated with lettuce) and F4546 (human isolate from an outbreak associated with alfalfa sprouts).

Preparation of inocula

The naturally occurring microflora on alfalfa seeds can interfere with the colony development of *Salmonella* and *E. coli* O157:H7 on traditional selective agar media. The resuscitation of heat-, desiccation- or chemically stressed cells of both pathogens may also be inhibited on these media. For these reasons, salmonellas and *E. coli* O157:H7 were grown in tryptic soy broth (pH 7·3; Difco, Detroit, MI, USA) supplemented with 50 μg nalidixic acid (Sigma Chemical, St. Louis, MO, USA) ml^{-1} (TSBN). Cultures were incubated at 37°C and transferred (1 loopful to 10 ml TSBN) three times at 24-h intervals immediately preceding use as inocula for alfalfa seeds. Populations of nalidixic acid-resistant pathogens in inocula were determined by serially diluting 24-h cultures in sterile 0·1% peptone and surface plating duplicate 0·1-ml samples on tryptic soy agar (Difco) supplemented with nalidixic acid (50 μg ml^{-1}; TSAN). Colonies that developed after 24 h at 37°C were counted.

Procedure for inoculating alfalfa seeds

A volume (6 ml) of 24-h TSBN cultures of each serotype of *Salmonella* was combined with 1 L sterile distilled water (22 ± 1°C) and mixed for 30 s. Alfalfa seeds (1 kg, 22 ± 1°C) were added to the diluted cell suspension and gently stirred for 1 min. The suspension containing the seeds was poured over a double layer of coarsely woven cloth supported by a wire screen elevated approximately 5 cm above the work surface of a laminar flow hood. The seeds were spread in a layer approximately 0·5 cm thick and allowed to dry for 72 ± 2 h in a hood at 22 ± 1°C before placing in plastic bags and storing at 5°C for 1 week. Previous observations on the viability of *Salmonella* and *E. coli* O157:H7 immediately after inoculation of alfalfa seed indicated that populations stabilized within 6–8 d at 5°C. Hence, seeds were stored for at least 1 week at 5°C to achieve populations with lower numbers of stressed cells before subjecting seeds to experiments designed to determine the lethality of temperature and/or chemical treatments.

Survival on dry seeds as affected by a_w

The influence of a_w on the survival of *Salmonella* and *E. coli* O157:H7 on alfalfa seeds stored at 5, 25 and 37°C for up to 52 weeks was determined. Inoculated seeds stored at 5°C for 1 week were distributed in a layer approximately 1 cm in depth over saturated solutions of magnesium chloride, magnesium nitrate or lithium chloride which

equilibrate with air above the solutions at relative humidities of 33, 52 and 68%, respectively. Seeds were stored at 5°C for 7 d. In addition, portions of seeds (a_w 0·15 and 0·21) inoculated with *E. coli* O157:H7 or *Salmonella*, respectively, were not exposed to atmospheres with these relative humidities.

Seeds (a_w 0·21–0·60) stored at 5, 25 and 37°C were monitored for populations of *Salmonella* for up to 42 weeks; seeds (a_w 0·15–0·54) stored at 5, 25 and 37°C were monitored for populations of *E. coli* O157:H7 for up to 52 weeks. Seeds (5 g) were combined with 20 ml Dey-Engley (DE) neutralizing broth (Difco) and stomached (Seward Medical, London, UK) for 30 s at medium speed. The DE wash broth was surface plated in quadruplicate (0·25 ml) or duplicate (0·1 ml) on TSAN. Duplicate 0·1-ml samples of DE wash broth serially diluted in 0·1% peptone were also plated on TSAN. Plates were incubated at 37°C for 24 h before presumptive colonies of pathogens were counted. Presumptive *Salmonella* colonies were randomly picked and tested for biochemical reactions using triple sugar iron (Difco) and lysine iron (Difco) agar slants. Presumptive *E. coli* O157:H7 colonies were confirmed using the *E. coli* O157 latex agglutination test (Oxoid, Basingstoke, Hampshire, UK) and API 20E diagnostic test (BioMerieux, Hazelwood, MO, USA).

Mixtures of 5 g seeds and 20 ml DE broth were also enriched for test pathogens. To the mixture containing seeds inoculated with *Salmonella*, 20 ml double-strength lactose broth (Difco) supplemented with 100 μg ml^{-1} nalidixic acid was added. After incubation for 24 h at 37°C, cultures (1 ml) were inoculated into 10 ml selenite cystine broth (Difco) and incubated for 24 h at 37°C. Selenite cystine broth cultures were streaked onto bismuth sulphite agar (Difco), supplemented with 50 μg ml^{-1} nalidixic acid and incubated for 24 h at 37°C before examination for presumptive *Salmonella* colonies. Randomly picked colonies were confirmed as *Salmonella* as described above. To the mixture of DE wash broth and seeds inoculated with *E. coli* O157:H7, 20 ml double-strength modified tryptic soy broth (Padhye and Doyle 1991) supplemented with 100 μg ml^{-1} nalidixic acid (mTSBN) was added. After incubation of the enrichment mixture at 37°C for 24 h, cultures were streaked on sorbitol MacConkey agar (Difco) supplemented with 50 μg ml^{-1} nalidixic acid and TSAN. Presumptive *E. coli* O157:H7 colonies formed after incubation of plates for 24 h at 37°C were confirmed as described above.

Seeds inoculated with *Salmonella* or *E. coli* O157:H7 and adjusted to various a_w values were also subjected to elevated temperatures known to be lethal to these pathogens. Seeds (a_w 0·25, 0·42 and 0·59) inoculated with *Salmonella* and seeds (a_w 0·15, 0·36 and 0·54) inoculated with *E. coli* O157:H7 were tested. Glass beakers (250 ml) were adjusted to 50, 60, 70 or 80°C in a forced-air oven and 5 g seeds were placed in an even layer in each beaker and heated for periods up to 24 h, depending on the temperature. At the end of the heat treatment, seeds were combined with 20 ml DE broth (22 ± 1°C) in a stomacher bag and analysed for the presence and populations of pathogens using procedures described above.

Combined effects of heat and aqueous chemical treatment

The effects of heating inoculated alfalfa seeds in aqueous chemical solutions on the inactivation of *Salmonella* and *E. coli* O157:H7 were studied. Initial experiments involved heating seeds (5 g) inoculated with *E. coli* O157:H7 in 20 ml tap water adjusted to 58, 61, 64 and 67°C for up to 10 min. Seeds were placed in a stomacher bag to which 20 ml water at each test temperature was added. The mixture of seeds and water was immersed in a water-bath at the desired test temperature and gently agitated for 2, 5 or 10 min. To the mixture of water and seeds, 20 ml mTSBN at 22 ± 1°C were added. The presence and/or number of *E. coli* O157:H7 remaining on the seeds were determined using procedures described above for the analysis of dry seeds.

Subsequent experiments were performed to determine the combined effects of heat and chemicals in the killing of *Salmonella* and *E. coli* O157:H7 on seeds. In addition to heating inoculated seeds in water, the following chemical treatments were evaluated: $Ca(OH)_2$ (1% (w/v) in deionized water; Sigma); Tween 80 (1% (v/v); Sigma); $Ca(OH)_2$ (1%) plus Tween 80 (1%) and Tsunami 200® (40 and 160 mg l^{-1}; Ecolab, Mendota Heights, MN, USA). Peroxyacetic acid concentrations in Tsunami solutions were determined using a Tsunami test kit (Ecolab). Seeds (5 g) inoculated with *Salmonella* were heated in water and chemical solutions (20 ml) at 23 and 55°C for 2, 5 and 10 min; seeds inoculated with *E. coli* O157:H7 were heated in water and chemical solutions at 23, 55 and 58°C for 2, 5 and 10 min. The procedures for detecting and/or enumerating pathogens on treated seeds after decanting water or chemical solutions and combining with 20 ml DE broth were as described above for dry seeds.

Determination of seed germination percentage

The germination percentage of untreated (control) and treated seeds was determined. Approximately 100 treated or control seeds were placed between two pieces of water-saturated filter paper (No. 4, 90 mm diameter; Whatman International, Maidstone, UK) in a Petri dish and placed in the dark at 30°C for 3 d, with periodic application of water. The number of germinated and ungerminated seeds was counted and the percentage that germinated calculated.

Table 1 Survival of *Salmonella* on alfalfa seeds as affected by temperature and a_w

Storage temp. (°C)	a_w	Storage time (weeks)	Population ($\log_{10}$ cfu g^{-1})*	Enrichment†	Reduction ($\log_{10}$ cfu g^{-1})‡	Germination (%)*
5	0·21	0	4·83 b			84·3 ab
		2	5·00 a		+ 0·17	85·1 ab
		12	4·53 bc		0·30	81·9 c
		26	4·76 bc		0·07	80·6 bc
		42	4·52 c		0·31	91·6 a
		52	4·60 bc		0·25	80·5 b
	0·40	0	4·83 a			83·7 b
		2	4·76 abc		0·07	86·7 ab
		12	4·66 bc		0·17	86·7 ab
		26	4·77 ab		0·06	89·3 a
		42	4·55 c		0·28	90·0 a
		52	4·63 bc		0·24	80·5 c
	0·60	0	4·77 a			85·1 b
		2	4·88 a		+ 0·11	77·8 c
		12	4·53 b		0·24	88·1 ab
		26	4·74 ab		0·03	84·7 b
		42	4·53 b		0·24	91·8 a
		52	4·51 bc		0·30	83·6 bc
25	0·21	0	4·83 a			84·3 ab
		2	4·78 a		0·05	86·6 a
		6	4·39 b		0·49	76·1 ab
		12	4·40 b		0·43	73·1 b
		26	4·09 b		0·74	74·4 b
		42	3·43 c		1·40	82·6 ab
		52	3·69 bc		1·15	79·7 abc
	0·40	0	4·83 a			83·7 a
		2	4·63 b		0·20	85·4 a
		6	3·92 c		0·91	84·1 a
		12	3·79 c		1·04	71·3 b
		26	3·53 c		1·30	77·7 ab
		42	3·88 c		0·95	78·9 ab
		52	3·64 c		1·23	76·8 ab
25	0·60	0	4·77 a			85·1 a
		2	4·05 b		0·72	77·0 a
		6	4·15 b		0·62	81·3 a
		12	3·62 b		1·15	72·8 a
		26	2·75 b		2·02	73·1 a
		42	1·41 b		3·36	71·9 a
		52	0·30 c	3	4·51	71·8 b
37	0·21	0	4·83 a			84·3 ab
		2	4·84 a		+ 0·01	86·6 ab
		4	4·30 b		0·53	76·7 b
		12	3·92 b		0·91	77·2 ab
		20	3·21 b		1·62	87·2 a
		42	2·39 b		2·44	84·0 ab
	0·40	0	4·83 a			83·7 a
		2	4·06 b		0·77	77·3 ab
		4	3·63 b		1·20	85·3 a
		12	2·42 b		2·41	73·7 b

Table 1 *Continued*

Storage temp. (°C)	a_w	Storage time (weeks)	Population ($\log_{10}$ cfu g^{-1})*	Enrichment†	Reduction ($\log_{10}$ cfu g^{-1})‡	Germination (%)*
		20	2·10 b		2·73	80·7 ab
		42	0§ b	3	4·83	74·7 b
	0·60	0	4·77 a			85·1 a
		2	3·40 b		1·37	73·4 b
		4	2·75 b		2·02	76·3 b
		12	0 b	3	4·77	48·5 c
		20	0 b	1	4·77	5·8 d
		42	0 b	0	4·77	0·7 d

*Within storage temperature and a_w, values in the same column that are not followed by the same letter are significantly different ($\alpha = 0{\cdot}05$).
†Number of samples of three analysed that were positive for *Salmonella* after enrichment.
‡Within storage temperature and a_w, $\log_{10}$ reduction in number of *Salmonella* compared with number initially on seeds (0 week).
§Values are < 1 cfu 4 g^{-1}.

Statistical analysis

All experiments were replicated three times. Data were subjected to the Statistical Analysis System (SAS Institute, Cary, NC, USA) for analysis of variance and Duncan's multiple range tests to determine differences ($\alpha = 0{\cdot}05$) in the populations of *Salmonella* or *E. coli* O157:H7 on alfalfa seeds as well as differences in the germination percentages of seeds as affected by treatment.

RESULTS

Long-term survival on dry seeds

Populations of *Salmonella* on alfalfa seeds stored for up to 52 weeks at 5 or 25°C, and up to 42 weeks at 37°C, as affected by a_w 0·21, 0·40 and 0·60, are shown in Table 1. In seeds with the same a_w, the rate of death increased as the storage temperature increased. The rate of death increased with an increase in the a_w of seeds stored at 25 or 37°C, but not at 5°C. Loss of seed viability followed the same general trends observed for loss of viability of *Salmonella*.

Table 2 shows the populations of *E. coli* O157:H7 detected on alfalfa seeds stored for up to 52 weeks as affected by the same temperatures and slightly different a_w (0·15, 0·36 and 0·54) as used in the study on the survival of *Salmonella* on dry alfalfa seeds. The correlations of increased temperature and a_w with increased rate of death of *E. coli* O157:H7 are similar to those observed for *Salmonella*. However, the adverse effects of elevated temperature and a_w on the viability of *E. coli* O157:H7, compared with *Salmonella*, were evident within a shorter storage time. Two different lots of seeds were used for the two storage studies. Although the germination percentage of seeds inoculated with *E. coli* O157:H7 (Table 2) was initially higher than that of seeds used in the study on the survival of *Salmonella* (Table 1), the loss of seed viability as affected by storage conditions in the two studies followed similar patterns.

Heat treatment of dry alfalfa seeds

The populations of *Salmonella* detected on alfalfa seeds adjusted to a_w 0·25, 0·42 and 0·59 and heated at 50, 60 and 70°C for up to 7 h are listed in Table 3. With the exception of seeds of a_w 0·59 heated at 70°C, heating at 50, 60 or 70°C for up to 24, 7 or 24 h, respectively, did not reduce populations by more than 0·61 $\log_{10}$ cfu g^{-1}. Treatment of seeds of a_w 0·59 at 70°C for 3 or 7 h reduced populations by 0·78 and 1·01 $\log_{10}$ cfu g^{-1}. The seed germination percentage was not greatly influenced by heat treatment.

Table 4 shows the results of experiments performed to determine the effects of a_w (0·15–0·54) on the inactivation of *E. coli* O157:H7 on seeds heated at 50, 60, 70 and 80°C for up to 24 h. The loss of viability of *E. coli* O157:H7 and *Salmonella* (Table 3) was similar when seeds were heated at 50°C for up to 24 h or 60°C for up to 2 h. However, when seeds were heated for 3 or 7 h at 60°C or ≥ 1 h at 70°C, reductions in viability of *E. coli* O157:H7 were much greater compared with those of *Salmonella*. At a given temperature, an increase in seed a_w generally enhanced the inactivation of *E. coli* O157:H7 (Table 4). For example, when seeds at a_w 0·35 or 0·52 were heated at 60°C for 7 or 3 h, respectively, or seeds at a_w 0·18, 0·35 or 0·52 were heated at 70°C for 3, 1 or 3 h, respectively, reductions of 1·04–1·91 $\log_{10}$ cfu g^{-1} occurred. Longer heating times resulted in reductions of up to 3·21 $\log_{10}$ cfu g^{-1}. Unfortunately, heating seeds at 60 or 70°C for ≥ 3 h or at 80°C for ≥ 1 h resulted in a decrease in the percentage seed germination.

Table 2 Survival of *Escherichia coli* O157:H7 on alfalfa seeds as affected by temperature and a_w

Storage temp. (°C)	a_w	Storage time (weeks)	Population ($\log_{10}$ cfu g^{-1})*	Enrichment†	Reduction ($\log_{10}$ cfu g^{-1})‡	Germination (%)*
5	0·15	0	3·18 b			92·9 a
		2	3·41 a		+ 0·23	91·3 ab
		12	3·00 c		0·18	80·0 c
		25	2·69 c		0·49	80·7 c
		52	2·57 c		0·61	83·6 bc
	0·36	0	3·25 a			91·6 a
		2	3·40 a		+ 0·15	91·3 a
		12	3·08 b		0·17	78·3 b
		25	2·86 b		0·39	83·4 b
		52	2·51 b		0·74	80·8 b
	0·54	0	2·93 a			92·3 ab
		2	3·00 a		+ 0·07	94·4 a
		12	2·75 b		0·18	81·8 c
		25	2·30 b		0·63	78·4 c
		52	2·00 b		0·93	83·1 bc
25	0·15	0	3·18 a			92·9 a
		2	2·78 b		0·40	93·9 a
		6	2·48 bc		0·70	87·6 a
		12	2·04 bc		1·14	72·7 b
		25	1·08 c		2·10	72·7 b
	0·36	0	3·25 a			91·6 a
		2	2·53 b		0·72	94·9 a
		6	1·89 b		1·36	81·5 b
		12	0·48 b	3	2·77	76·9 bc
		25	0·30 b	3	2·95	70·3 c
	0·54	0	2·93 a			92·3 a
		2	1·65 b	3	1·28	93·1 a
		6	1·57 b	3	1·36	82·0 b
		12	0§ b	3	2·93	77·8 b
		25	0 b	3	2·93	77·8 b
37	0·15	0	3·18 a			92·9 a
		2	2·20 b		0·98	94·1 a
		4	0 c	3	3·18	95·3 a
		8	0 c	1	3·18	74·9 c
		14	0 c	0	3·18	80·0 b
	0·36	0	3·25 a			91·6 a
		2	1·96 b		1·29	92·3 a
		4	0·70 b	3	2·55	96·5 a
		8	0 b	1	3·25	75·8 b
		14	0 b	0	3·25	80·9 b
	0·54	0	2·93 a			92·3 b
		2	0·70 b	3	2·23	93·1 ab
		4	0 b	1	2·93	97·2 a
		8	0 b	0	2·93	81·6 c
		14	0 b	0	2·93	71·8 d

*Within storage temperature and a_w, values in the same column that are not followed by the same letter are significantly different ($\alpha = 0{\cdot}05$).
†Number of samples of three analysed that were positive for *E. coli* O157:H7 after enrichment.
‡Within storage temperature and a_w, $\log_{10}$ reduction in number of *E. coli* O157:H7 compared with number initially on seeds (0 week).
§Values are < 1 cfu 4 g^{-1}.

Table 3 Effect of dry heat treatment in killing *Salmonella* on alfalfa seeds at a_w 0·25–0·59

Treatment temp. (°C)	Initial a_w	Treatment time (h)	Population ($\log_{10}$ cfu g^{-1})*	Reduction ($\log_{10}$ cfu g^{-1})†	Germination (%)*
50	0·25	0	5·05 a		88·3 a
		3	4·78 b	0·27	87·3 ab
		7	4·74 c	0·31	83·6 b
		24	4·44 c	0·61	86·9 ab
	0·42	0	4·81 a		87·8 a
		3	4·71 a	0·10	86·7 a
		7	4·61 a	0·20	86·0 a
		24	4·62 a	0·19	91·4 a
	0·59	0	4·52 b		86·3 a
		3	4·41 b	0·11	72·8 b
		7	4·56 b	+ 0·04	86·9 a
		24	4·62 a	+ 0·30	81·6 a
60‡	0·25	0	5·05 a		87·3 ab
		0·5	4·62 c	0·43	84·4 b
		1	4·82 b	0·23	86·9 ab
		2	4·56 c	0·49	91·7 a
	0·42	0	4·81 a		87·8 a
		0·5	4·44 b	0·37	87·2 a
		1	4·55 ab	0·26	91·0 a
		2	4·39 b	0·42	91·3 a
	0·59	0	4·52 a		86·2 b
		0·5	4·32 b	0·20	86·6 ab
		1	4·49 a	0·03	90·2 a
		2	4·49 a	0·03	88·7 ab
60§	0·25	0	4·91 a		81·9 a
		1	4·45 b	0·46	87·1 a
		3	4·51 b	0·40	82·4 a
		7	4·47 b	0·44	81·8 a
	0·42	0	4·80 a		86·0 a
		1	4·52 b	0·28	90·0 a
		3	4·46 b	0·34	89·4 a
		7	4·26 c	0·54	76·4 b
	0·59	0	4·78 a		85·0 ab
		1	4·34 b	0·44	82·1 b
		3	4·29 b	0·49	87·6 a
		7	4·20 c	0·58	77·4 c
70	0·25	0	4·78 a		93·3 a
		1	4·31 b	0·47	89·8 a
		3	4·51 b	0·27	92·8 a
		7	4·40 b	0·38	85·7 b
	0·42	0	4·65 a		86·5 bc
		1	4·40 ab	0·25	82·0 c
		3	4·28 b	0·37	93·9 a
		7	4·10 b	0·55	88·8 b
	0·59	0	4·85 a		86·0 b
		1	4·23 b	0·62	79·5 c
		3	4·07 b	0·78	83·6 a
		7	3·84 b	1·01	83·1 bc

*Within treatment temperature (60°C‡ and 60°C§ treatments were analysed separately) and a_w, values in the same column that are not followed by the same letter are significantly different ($\alpha = 0{\cdot}05$).

†Within treatment temperature (60°C‡ and 60°C§ treatments were analysed separately) and a_w, $\log_{10}$ reduction in number of *Salmonella* compared with no heat treatment (0 h).

‡,§Two separate experiments using a treatment temperature of 60°C were performed.

Table 4 Effect of dry heat treatment in killing *Escherichia coli* O157:H7 on alfalfa seeds at a_w 0·15–0·54

Treatment temp. (°C)	Initial a_w	Treatment time (h)	Population ($\log_{10}$ cfu g^{-1})*	Enrichment†	Reduction ($\log_{10}$ cfu g^{-1})‡	Germination (%)*
50	0·15	0	3·19 a			92·5 b
		3	3·09 b		0·10	96·1 a
		7	3·12 b		0·07	93·0 ab
		24	2·78 c		0·31	94·2 ab
	0·36	0	3·26 a			91·6 c
		3	3·14 b		0·12	97·6 a
		7	3·04 b		0·22	94·9 b
		24	2·82 c		0·44	94·5 b
	0·54	0	2·93 a			92·3 b
		3	2·93 a		0	97·2 a
		7	2·68 b		0·25	94·5 ab
		24	2·24 c		0·69	94·5 ab
60§	0·15	0	3·18 a			92·9 c
		0·5	3·15 ab		0·03	94·4 bc
		1	3·04 bc		0·14	95·5 ab
		2	2·96 c		0·22	96·6 a
	0·36	0	3·26 a			91·6 b
		0·5	3·10 bc		0·16	95·5 ab
		1	3·17 ab		0·09	96·0 a
		2	3·03 c		0·23	97·1 a
	0·54	0	2·93 a			92·3 c
		0·5	2·60 b		0·33	95·6 b
		1	2·38 c		0·55	97·2 a
		2	2·65 b		0·28	96·0 ab
60**	0·18	0	3·73 a			88·5 a
		1	3·53 b		0·20	89·2 a
		3	3·10 c		0·63	82·1 b
		7	2·79 d		0·94	70·7 c
	0·35	0	3·80 a			85·2 b
		1	3·53 b		0·27	88·9 a
		3	2·84 c		0·96	85·5 b
		7	2·43 d		1·37	72·9 c
	0·52	0	3·49 a			87·4 a
		1	2·72 b		0·77	87·5 a
		3	2·31 c		1·18	84·5 ab
		7	1·19 d		2·30	81·0 b
70	0·18	0	3·64 a			93·2 b
		1	2·73 b		0·91	98·2 a
		3	2·60 b		1·04	87·4 c
		7	2·12 c		1·52	81·4 d
	0·35	0	3·60 a			97·3 a
		1	1·69 b		1·91	98·6 b
		3	1·83 b		1·77	89·5 b
		7	0·39 b	3	3·21	84·6 c
	0·52	0	3·44 a			96·9 b
		1	2·57 b		0·87	99·2 a
		3	1·67 c		1·77	89·7 c

Table 4 *Continued*

Treatment temp. (°C)	Initial a_w	Treatment time (h)	Population ($\log_{10}$ cfu g^{-1})*	Enrichment†	Reduction ($\log_{10}$ cfu g^{-1})‡	Germination (%)*
		7	0·63 d	3	2·81	86·8 d
80	0·18	0	3·64 a			93·2 a
		1	2·25 b		1·39	98·6 b
		3	1·44 c		2·20	57·1 c
		7	1·39 c		2·25	22·9 c
	0·35	0	3·60 a			97·3 a
		1	2·01 b		1·59	94·8 a
		3	1·08 c		2·52	51·6 b
		7	0·67 c	3	2·93	19·4 c
	0·52	0	3·44 a			96·9 a
		1	1·56 b		1·88	83·6 b
		3	0¶ c	0	3·44	16·3 c
		7	0 c	0	3·44	18·1 c

*Within treatment temperature (60°C§ and 60°C** treatments were analysed separately) and a_w, values in the same column that are not followed by the same letter are significantly different ($\alpha = 0{\cdot}05$).

†Number of samples out of three analysed that were positive for *E. coli* O157:H7 after enrichment.

‡Within treatment temperature (60°C§ and 60°C** treatments were analysed separately) and a_w, $\log_{10}$ reduction in number of *E. coli* O157:H7 compared with no heat treatment (0 h).

§,**Two separate experiments using a treatment temperature of 60°C were performed.

¶Values are < 1 cfu 4 g^{-1}.

Combined effects of heat and chemical treatment

The results of experiments to determine the effects of heating alfalfa seeds in water and chemical solutions at 23 and 55°C on the inactivation of *Salmonella* are summarized in Table 5. Treatment at 55°C, compared with 23°C, enhanced the inactivation of *Salmonella*. Treatment with water, 1% $Ca(OH)_2$, 1% Tween 80, 1% $Ca(OH)_2$ plus 1% Tween 80 or 40 or 160 mg l^{-1} Tsunami 200 at 23 or 55°C significantly reduced populations of the pathogen. Treatment at 55°C resulted in greater reductions than treatment at 23°C. Treatment with chemicals at 23°C for up to 10 min resulted in reductions in populations of *Salmonella* of 1·35–2·64 $\log_{10}$ cfu g^{-1} without substantial loss in seed viability. Treatment at 55°C significantly increased the number of *Salmonella* removed from seeds or killed but, in some instances, also reduced the percentage seed germination. Overall, treatment with 1% $Ca(OH)_2$ was most effective in killing *Salmonella* without sacrificing seed viability.

The inactivation of *E. coli* O157:H7 on dry (a_w 0·72) alfalfa seeds heated at 58–67°C (Table 6) was determined before testing the efficacy of chemical treatment at elevated temperatures. Heating at 58°C for 5 min or ≥ 61°C for 2 min significantly reduced the population of *E. coli* O157:H7. Treatment at 58, 61 or 64°C for ≥ 5 min or 67°C for ≥ 2 min significantly reduced the percentage seed germination and, with the exception of treatment at 67°C for 10 min, did not eliminate the pathogen.

Treatment temperatures of 55 and 58°C were chosen for subsequent experiments to determine the combined effects of heat and chemicals on the inactivation of *E. coli* O157:H7 on alfalfa seeds. Results are shown in Table 7. In the first experiment, seeds were heated only at 58°C. Treatment for 2 min in water or a solution of 1% $Ca(OH)_2$, with or without 1% Tween 80, resulted in significant reductions in populations. Reductions of ≥ 2·24 $\log_{10}$ cfu g^{-1} occurred in seeds heated in 1% $Ca(OH)_2$ for 2 min without a significant reduction in the percentage seed germination. A second experiment was performed to determine the efficacy of Tsunami 200 in killing *E. coli* O157:H7 on seeds held at 23 and 58°C. The treatment of seeds for 10 min at 23°C with 40 or 60 mg l^{-1} Tsunami 200 resulted in reductions in populations of 1·12 and 1·30 $\log_{10}$ cfu g^{-1}, respectively, without loss of seed viability. Treatment with 40 or 160 mg l^{-1} Tsunami 200 for 5 min at 58°C resulted in population decreases of 2·88 and 2·46 $\log_{10}$ cfu g^{-1}, respectively, without a significant decrease in the percentage seed germination. In the third experiment, the lethality of $Ca(OH)_2$, $Ca(OH)_2$ plus Tween 80 and Tsunami 200 at 55°C was tested. Compared with treatment in water or Tsunami at 58°C (second experiment), the reductions in populations of *E. coli* O157:H7 were less when seeds were treated at 55°C. Reductions of 1·68 and 1·27 $\log_{10}$ cfu g^{-1} on

Table 5 Effect of chemical treatment at 23 and 55°C in killing *Salmonella* on alfalfa seeds

Chemical	Treatment		Population (log_{10} cfu g^{-1})*	Reduction (log_{10} cfu g^{-1})†	Germination (%)*
	Temp. (°C)	Time (min)			
Water	23	0	4·83 a		88·3 a
		2	4·37 b	0·46	89·4 a
		5	4·10 c	0·73	87·2 a
		10	4·07 c	0·76	91·8 a
	55	0	4·83 a		88·3 a
		2	3·38 b	1·45	88·2 a
		5	2·82 c	2·01	86·6 a
		10	2·24 e	2·59	66·5 b
$Ca(OH)_2$ (1%)	23	0	4·83 a		88·3 a
		2	2·23 b	2·60	92·4 a
		5	2·63 b	2·20	92·5 a
		10	2·19 b	2·64	91·3 a
	55	0	4·83 a		88·3 a
		2	1·69 b	3·14	78·8 b
		5	0·47 c	4·36	88·4 a
		10	0·43 c	4·40	56·8 c
Tween 80 (1%)	23	0	4·78 a		88·9 a
		2	4·22 b	0·56	92·0 a
		5	4·31 b	0·47	77·1 b
		10	4·02 c	0·76	66·2 c
	55	0	4·78 a		88·9 a
		2	3·42 b	1·36	68·8 bc
		5	2·75 c	2·03	76·7 ab
		10	2·28 d	2·50	60·2 c
$Ca(OH)_2$ (1%) + Tween 80 (1%)	23	0	4·83 a		88·3 a
		2	2·83 c	2·00	86·7 a
		5	3·12 b	1·71	87·5 a
		10	3·05 b	1·78	88·3 a
	55	0	4·83 a		88·3 a
		2	1·64 b	3·19	80·8 b
		5	1·15 c	3·68	73·9 c
		10	0·23 d	4·60	72·5 c
Tsunami 200 (40 mg l^{-1})	23	0	4·78 a		88·9 a
		2	3·04 b	1·74	93·1 a
		5	3·43 b	1·35	89·7 a
		10	3·27 b	1·51	91·8 a
	55	0	4·78 a		88·9 ab
		2	2·95 b	1·83	91·2 a
		5	2·30 c	2·48	83·8 b
		10	1·70 d	3·08	84·0 b
Tsunami 200 (160 mg l^{-1})	23	0	4·78 a		88·9 b
		2	2·73 c	2·05	91·3 ab
		5	3·08 b	1·70	91·3 ab
		10	3·14 b	1·60	92·4 a
	55	0	4·78 a		88·9 a
		2	2·53 b	2·25	93·6 a
		5	2·44 b	2·34	92·7 a
		10	1·63 c	3·15	74·2 b

*Within chemical treatment and temperature, values in the same column that are not followed by the same letter are significantly different ($\alpha = 0{\cdot}05$).
†Within chemical treatment and temperature, log_{10} reduction in number of *Salmonella* compared with no treatment (0 min).

Table 6 Effect of dry heat treatment in killing *Escherichia coli* O157:H7 on alfalfa seeds

Treatment					
Temp. (°C)	Time (min)	Population ($\log_{10}$ cfu g^{-1})*	Enrichment†	Reduction ($\log_{10}$ cfu g^{-1})‡	Germination (%)*
58	0	2·79 a			82·2 a
	2	2·18 ab		0·61	79·7 a
	5	1·08 bc	3	1·71	62·3 b
	10	0·32 c	3	2·47	49·3 c
61	0	2·79 a			82·2 a
	2	0·80 b	3	1·99	74·4 ab
	5	0§ c	3	2·79	67·4 b
	10	0 c	3	2·79	36·4 c
64	0	2·79 a			82·2 a
	2	0·32 b	3	2·47	75·7 a
	5	0 b	3	2·79	64·9 b
	10	0 b	1	2·79	26·0 c
67	0	2·79 a			82·2 a
	2	0 b	3	2·79	69·1 b
	5	0 b	3	2·79	36·9 c
	10	0 b	0	2·79	15·9 d

*Within treatment temperature, values in the same column that are not followed by the same letter are significantly different ($\alpha = 0{\cdot}05$).
†Number of samples out of three analysed that were positive for *E. coli* O157:H7 after enrichment.
‡Within treatment temperature, $\log_{10}$ reduction in number of *E. coli* O157:H7 compared with no heat treatment (0 min).
§Values are < 1 cfu 4 g^{-1}.

seeds treated with 1% $Ca(OH)_2$ for 10 min or 40 mg l^{-1} Tsunami 200 for 5 min, respectively, were achieved without significant reductions in seed viability.

DISCUSSION

Observations on the survival of *Salmonella* on alfalfa seeds stored at 5, 25 and 37°C, as affected by a_w, are in agreement with other reports describing the rates of death of the organism in dry foods. Christian and Stewart (1973) observed that low a_w protected *Salm.* Newport against inactivation in cake mix, skim milk, onion soup and flummery stored at 25°C. Juven *et al.* (1984) reported that the survival of *Salm.* Heidelberg and *Salm.* Montevideo was enhanced in dry milk and cocoa powder. The results of our study are also in agreement with those of Jaquette *et al.* (1996), who reported that populations of *Salm.* Stanley on dry alfalfa seeds held at 8°C for 9 weeks or 8°C for 1 week, then 21°C for 8 weeks, were reduced by 0·72 and 1·61 $\log_{10}$ cfu g^{-1}, respectively. Neither the moisture content nor the a_w of the seeds was reported.

Retention of viability of *E. coli* O157:H7 on alfalfa seeds was enhanced when seeds were stored at 5°C compared with storage at 25 or 37°C and, within storage temperature, as the a_w was reduced. Taormina and Beuchat (1999) monitored populations of *E. coli* O157:H7 on alfalfa seeds (5·7 ± 0·5% moisture) stored at 5, 25 and 37°C for up to 54 weeks. The rates of inactivation were similar to those observed for seeds at a_w 0·54 in the present study. A set of seed samples originally inoculated with 3·04 $\log_{10}$ cfu g^{-1} in the study of Taormina and Beuchat (1999) was analysed for the presence of *E. coli* O157:H7 after storage for 164 weeks at 5°C and found to be positive for the pathogen. Like *Salmonella*, the survival of *E. coli* O157:H7 on alfalfa seed and in other dry foods (Ryu *et al.* 1999; Park and Beuchat 2000) is enhanced as the a_w and storage temperature are decreased.

Although the rate of inactivation of *Salmonella* and *E. coli* O157:H7 on alfalfa seeds is increased by increasing the a_w and temperature, these conditions unfortunately also adversely affect seed viability. It does not appear that a combination of a_w and temperature conditions can be selected to eliminate the pathogens and simultaneously retain viability of seeds.

Initial studies to evaluate the efficacy of sanitizers in killing *Salmonella* on alfalfa seeds clearly revealed the level of difficulty in achieving success (Beuchat 1997; Jaquette *et al.* 1996). Subsequent studies demonstrated that treatment with 20 000 mg l^{-1} active chlorine [as $Ca(OCl)_2$] failed to kill *E. coli* O157:H7 on seeds containing 2·68 $\log_{10}$ cfu g^{-1} (Taormina and Beuchat 1999). Lang *et al.* (2000) were

Table 7 Effect of chemical treatment at 23–58°C in killing *Escherichia coli* O157:H7 on alfalfa seeds

Experiment/ chemical	Treatment		Population (log_{10} cfu g^{-1})*	Enrichment†	Reduction (log_{10} cfu g^{-1})‡	Germination (%)*
	Temp. (°C)	Time (min)				
Experiment 1						
Water	58	0	3·68 a			82·5 a
		2	2·30 b		1·38	82·1 a
		5	1·82 b		1·86	75·4 a
		10	0·67 c	3	3·01	44·4 b
$Ca(OH)_2$ (1%)	58	0	3·68 a			82·5 a
(pH 12·8)		2	1·44 b		2·24	72·1 ab
		5	1·43 b		2·25	60·4 bc
		10	0§ c	3	3·68	48·5 c
$Ca(OH)_2$ (1%) +	58	0	3·68 a			82·5 a
Tween 80 (1%)		2	1·34 b		2·34	77·8 ab
(pH 12·4)		5	0 c	3	3·68	71·0 b
		10	0 c	3	3·68	44·1 c
Experiment 2						
Water	23	0	2·88 a			79·3 a
		2	2·62 ab		0·26	74·3 a
		5	2·51 bc		0·37	79·6 a
		10	2·25 c		0·62	81·7 a
	58	0	2·88 a			79·3 a
		2	1·21 b		1·67	77·4 ab
		5	0·32 c	3	2·56	68·6 b
		10	0 c	3	2·88	69·6 b
Tsunami 200	23	0	2·88 a			79·3 a
(40 mg l^{-1})		2	2·22 b		0·66	83·4 a
(pH 2·8)		5	1·76 b		1·12	80·2 a
		10	1·73 b		1·12	83·9 a
	58	0	2·88 a			79·3 a
		2	1·00 b		1·88	80·3 a
		5	0 c	3	2·88	74·2 a
		10	0 c	3	2·88	57·1 b
Tsunami 200	23	0	2·88 a			79·3 a
(160 mg l^{-1})		2	1·92 ab		0·96	81·6 a
(pH 2·8)		5	1·84 b		1·04	80·5 a
		10	0·58 c	3	1·30	80·1 a
	58	0	2·88 a			79·3 a
		2	1·19 b		1·69	82·1 a
		5	0·42 b	3	2·46	80·1 a
		10	0·32 b	3	2·56	58·3 b
Experiment 3						
Water	55	0	2·63 a			82·3 ab
		2	2·30 b		0·33	85·5 a
		5	1·49 c		1·14	76·2 bc
		10	1·15 d		1·48	67·7 c
$Ca(OH)_2$ (1%)	55	0	2·63 a			82·3 a
(pH 12·8)		2	1·71 b		0·92	77·9 a
		5	1·29 b		1·34	74·3 a

Table 7 *Continued*

Experiment/ chemical	Treatment Temp. (°C)	Time (min)	Population (log_{10} cfu g^{-1})*	Enrichment†	Reduction (log_{10} cfu g^{-1})‡	Germination (%)*
		10	0·95 b		1·68	73·0 a
$Ca(OH)_2$ (1%) +	55	0	2·63 a			82·3 a
Tween 80 (1%)		2	1·66 b		0·97	82·2 a
(pH 12·4)		5	1·55 b		1·08	76·9 a
		10	1·53 b		1·10	74·0 a
Tsunami 200	55	0	2·63 a			82·3 a
(40 mg l^{-1})		2	1·73 b		0·90	80·5 a
(pH 2·8)		5	1·36 b		1·27	75·9 a
		10	0·84 b		1·79	62·2 b
Tsunami 200	55	0	2·63 a			82·3 a
(160 mg l^{-1})		2	1·73 b		0·90	85·3 a
(pH 2·8)		5	0·84 c		1·79	72·5 b
		10	0 d	3	2·63	48·5 c

*Within each experiment, chemical treatment and temperature, values in the same column that are not followed by the same letter are significantly different ($\alpha = 0{\cdot}05$).

†Number of samples of three analysed that were positive for *E. coli* O157:H7 after enrichment.

‡Within each experiment, chemical treatment and temperature, log_{10} reduction in number of *E. coli* O157:H7 compared with no treatment (0 min).

§Values are < 1 cfu 4 g^{-1}.

successful in reducing *E. coli* O157:H7 populations by 2–4 log cfu g^{-1} of alfalfa seeds using successive applications of lactic acid and hypochlorite but did not eliminate the pathogen, as evidenced by its presence on sprouts produced from the treated seeds.

Other recent studies have shown that treatment with a liquid prototype produce wash (Fit®) compared favourably with 20 000 mg l^{-1} chlorine in killing *Salmonella* and *E. coli* O157:H7 on alfalfa seeds (Beuchat *et al*. 2001). Among various concentrations of chemicals investigated by Weissinger and Beuchat (2000), the treatment of alfalfa seeds at 23°C with 20 000 mg l^{-1} chlorine, 5% trisodium phosphate, 8% hydrogen peroxide, 1% $Ca(OH)_2$, 1% calcinated calcium, 5% lactic acid or 5% citric resulted in reductions in *Salmonella* populations of 2·0–3·2 log_{10} cfu g^{-1}. With the exceptions of 8% hydrogen peroxide, 1% $Ca(OH)_2$ and 1% calcinated calcium that reduced populations by 3·2, 2·8 and 2·9 log_{10} cfu g^{-1}, respectively, none of the treatments reduced the number of *Salmonella* by more than 2·2 log_{10} cfu g^{-1} without significantly reducing the percentage seed germination. Observations reported here on the effectiveness of 1% $Ca(OH)_2$ in killing *Salmonella* on seeds at 23°C are in agreement with this. Treatment at 55°C significantly increased the number of *Salmonella* killed or removed from seeds but also reduced the percentage seed germination.

As with simultaneous interactions of a_w and temperature that increase the rate of inactivation of *Salmonella* and *E. coli* O157:H7 but also adversely affect seed viability, the combined effects of either a_w or chemicals with heat to kill these pathogens also results in loss of seed viability. A highly effective treatment or combination of treatments that can legally be applied to eliminate *Salmonella* and *E. coli* O157:H7 on alfalfa seed without compromising seed viability has yet to be developed and validated. The US Food and Drug Administration currently recommends that seeds intended for sprout production be treated by immersing in a 20 000 mg l^{-1} calcium hypochlorite solution. This procedure raises concern over worker safety and creates expense associated with disposal of the treatment solution. The use of high concentrations of trisodium phosphate, hydrogen peroxide or even 1% $Ca(OH)_2$ is not without potential hazards to the health of workers. The use of 1% $Ca(OH)_2$ as a disinfectant, however, may be less hazardous than 20 000 mg l^{-1} calcium hypochlorite. Given the lack of a validated system to disinfect alfalfa seeds, it is essential that practices to prevent contamination of seeds with human pathogens be strictly followed in order to minimize the risk of infections associated with sprouts produced from these seeds.

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