

Efficacy of Chlorine and Heat Treatment in Killing *Salmonella stanley* Inoculated onto Alfalfa Seeds and Growth and Survival of the Pathogen during Sprouting and Storage

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The efficacy of chlorine and hot water treatments in killing *Salmonella stanley* inoculated onto alfalfa seeds was determined. Treatment of seeds containing 10^2 to 10^3 CFU/g in 100- μ g/ml active chlorine solution for 5 or 10 min caused a significant ($P \leq 0.05$) reduction in population, and treatment in 290- μ g/ml chlorine solution resulted in a significant reduction compared with treatment in 100 μ g of chlorine per ml. However, concentrations of chlorine of up to 1,010 μ g/ml failed to result in further significant reductions. Treatment of seeds containing 10^1 to 10^2 CFU of *S. stanley* per g for 5 min in a solution containing 2,040 μ g of chlorine per ml reduced the population to undetectable levels (<1 CFU/g). Treatment of seeds in water for 5 or 10 min at 54°C caused a significant reduction in the *S. stanley* population, and treatment at $\geq 57^\circ\text{C}$ reduced populations to ≤ 1 CFU/g. However, treatment at $\geq 54^\circ\text{C}$ for 10 min caused a substantial reduction in viability of the seeds. Treatment at 57 or 60°C for 5 min appears to be effective in killing *S. stanley* without substantially decreasing germinability of seeds. Storage of seeds for 8 to 9 weeks at 8 and 21°C resulted in reductions in populations of *S. stanley* of about 1 \log_{10} and 2 \log_{10} CFU/g, respectively. The behavior of *S. stanley* on seeds during soaking, germination, sprouting, and refrigerated storage of sprouts was determined. An initial population of 3.29 \log_{10} CFU/g increased slightly during 6 h of soaking, by about 10^3 CFU/g during a 24-h germination period, and by an additional 10 CFU/g during a 72-h sprouting stage. A population of 10^7 CFU/g of mature alfalfa sprouts was detected throughout a subsequent 10-day storage period at 5°C. These studies indicate that while populations of *S. stanley* can be greatly reduced, elimination of this organism from alfalfa seeds may not be reliably achieved with traditional disinfection procedures. If *S. stanley* is present on seeds at the initiation of the sprout production process, populations exceeding 10^7 CFU/g can develop and survive on mature sprouts exposed to handling practices used in commercial production and marketing.

Poultry, eggs, and dairy products are the most commonly implicated sources of salmonellosis outbreaks (13). However, salmonellae have been isolated from several types of fresh vegetables, some of which have been linked to human salmonellosis (3). Salmonellae have been isolated from lettuce and fennel in Italy (6) and numerous salad vegetables in Spain (8). Two multistate outbreaks of salmonellosis in the United States have been linked to the consumption of raw tomatoes (9, 14).

A survey in Thailand revealed the presence of several serotypes of salmonellae on bean sprouts (10), and an outbreak of 143 cases of *Salmonella saint-paul* infection in the United Kingdom was associated with the consumption of mung bean sprouts (11). *Salmonella bovismorbificans* was cultured from alfalfa sprouts implicated in an outbreak in Finland (12), and *Salmonella anatum* infection has been linked to alfalfa sprouts in the United States (5a). In 1995, an outbreak of *Salmonella stanley* infections associated with the consumption of alfalfa sprouts affected persons in at least 17 states and in Finland (5a). The presence of *Salmonella* cells on alfalfa seeds (2) and their ability to grow on mung beans and alfalfa seeds during sprouting (1) raise concern about procedures used to handle seeds and conditions affecting survival and growth of salmonellae during the sprouting process and subsequent storage before consumption.

The study reported here was undertaken to determine the

efficacy of chlorine and heat treatment in killing *S. stanley* inoculated onto alfalfa seeds. Retention of viability of *S. stanley* on dry seeds as affected by storage temperature was also investigated. A third objective was to determine the behavior of *S. stanley* on alfalfa seeds subjected to conditions used commercially to grow and market sprouts.

MATERIALS AND METHODS

Strain and preparation of inoculum. *S. stanley* H0558 was used throughout the study. This strain was isolated from a patient involved in the 1995 outbreak of salmonellosis linked to alfalfa sprouts. A stock culture was maintained on tryptic soy agar (Difco, Detroit, Mich.) at 5°C. The organism was cultured in tryptic soy broth (pH 7.3; Difco) at 37°C. Loop inocula were transferred to tryptic soy broth at three consecutive 24-h intervals immediately before use as inocula for experiments.

Procedure for inoculating alfalfa seeds. Alfalfa seeds were obtained from Caudill Seed Company, Louisville, Ky. Sterile deionized water (3 liters) was combined with 30 ml of a 24-h tryptic soy broth culture of *S. stanley* or 30 ml of a culture diluted 10^{-2} in sterile 0.1% peptone to yield two populations in dip suspensions. Three kilograms of seed was combined with each cell suspension, and the suspensions were gently mixed for 1 min. The suspensions were decanted, and seeds were placed on wire screens lined with cheesecloth to dry under a laminar flow hood for 24 h. Seeds were then placed in plastic bags and held at 21°C for 48 h before being stored at 8°C until used in chlorine and heat treatment experiments. A portion of the inoculated seeds was stored at 8°C for 9 weeks or at 8°C for 1 week and subsequently at 21°C for 8 weeks to determine the survival of *S. stanley* at temperatures at which seeds might be stored in commercial settings.

Chlorine and hot water treatments. Inactivation of *S. stanley* on alfalfa seeds dipped in solutions containing target concentrations of 0, 100, 300, 500, 1,000, 2,000, and 4,000 μ g of active chlorine per ml was determined. Inoculated seeds were stored at 8°C; seeds were stored for 5 days for studies using 100 to 1,000 μ g of active chlorine per ml and for 9 weeks for studies using 2,000 and 4,000 μ g of

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chlorine per ml. Chlorine solutions (21°C) were prepared by adding appropriate amounts of 5% sodium hypochlorite (Aldrich, Milwaukee, Wis.) to 0.05 M potassium phosphate buffer (pH 6.8; 21°C). The free chlorine content of dip solutions was determined with Hach Co. (Ames, Iowa) chlorine test kits, which are approved by the U.S. Environmental Protection Agency. Seeds (25 g) were combined with 100 ml of chlorine solution in a stomacher bag and soaked for 5 or 10 min, with occasional agitation. Samples of the soak solution were withdrawn and analyzed for populations of *S. stanley*, and the remaining soak solution was decanted. Fifty milliliters of sterile 0.1% peptone was then added to the seeds, and the mixture was pummeled in a stomacher for 30 s at medium speed. Chlorine soak solutions and peptone wash solutions were serially diluted and surface plated (0.1 ml) in duplicate on bismuth sulfite agar (BSA) (Difco). Undiluted samples (0.25 ml) were also plated in quadruplicate on BSA. Presumptive *S. stanley* colonies formed on plates incubated at 37°C for 24 h were counted. Colonies were randomly selected and confirmed by appropriate biochemical tests (7).

Inactivation of *S. stanley* on seeds as affected by dipping in hot water for 5 or 10 min was determined. Sterile tap water (150 ml) tempered in a water bath at 21, 54, 57, 60, 63, 66, or 71°C was added to 25 g of inoculated seeds (21°C) in stomacher bags and held at desired temperatures, with occasional agitation, for 5 or 10 min. Duplicate 0.1-ml or quadruplicate 0.25-ml samples of dip water were surface plated on BSA, and then the remaining water was decanted and 50 ml of sterile 0.1% peptone was added to the treated seeds. The mixture was pummeled for 30 s at medium speed, and then duplicate 0.1-ml and quadruplicate 0.25-ml samples of wash solution were surface plated on BSA. Presumptive *S. stanley* colonies were counted after incubation at 37°C for 24 h and were confirmed by biochemical tests.

Germination of seeds as affected by hot water treatments. Uninoculated seeds treated with hot water as described above were placed between two sterile moistened filter pads in sterile petri plates. The percentage of seeds that germinated after 48 h at 30°C was determined.

Survival of *S. stanley* on dry seeds. Inoculated seeds stored either at 8°C for 9 weeks or at 8°C for 1 week and subsequently at 21°C for 8 weeks were examined for populations of *S. stanley*. The procedure for analysis was the same as that used for determining populations of *S. stanley* on seeds treated with chlorine and hot water.

Behavior of *S. stanley* during sprouting and storage. Inoculated seeds stored at 8°C for 24 days were used to determine the behavior of *S. stanley* during sprouting and under subsequent refrigerated storage conditions simulating those used commercially. Inoculated seeds and sterile tap water were combined at a 1:4 (wt/vol) ratio, and the seeds were soaked, with occasional mixing, for 6 h at 21°C. Seeds were drained, spread in 0.5-cm-thick layers on water-saturated absorbent cotton covered with cheesecloth, placed under fluorescent light, and incubated for 24 h at 21°C. Fifty-gram samples of germinated seeds were then placed on sterile water-saturated filter pads in plastic containers (base dimensions, 8 by 8 cm; height, 6.5 cm; top, 10 by 10 cm). Containers were set on water-saturated cotton and placed under fluorescent light at 21°C for 3 days. Water was sprayed on the surface of seeds and sprouts at 3- to 6-h intervals during germination and sprouting to maintain moisture conditions necessary for the development of mature sprouts. At the end of the sprouting process (4.25 days from initiation of soaking), sprouts were placed in a refrigerator at 5°C for 10 days. Samples of soak water and soaked seeds, as well as samples at various stages of germination, sprout development, and refrigerated storage, were analyzed for populations of *S. stanley*. Except for dry seeds, of which 25-g samples were analyzed as described above, 50-g samples were analyzed during the soaking, germinating, and sprouting stages and during subsequent refrigeration; samples were combined with 100 ml of sterile 0.1% peptone water and washed by gentle hand massaging for 1 min. Caution was taken to not break sprouts, as breakage might have resulted in the release of phytoalexins inhibitory to *S. stanley*. The wash solution was surface plated on BSA to enumerate *S. stanley* cells as described above. Populations of *S. stanley* per gram of seeds or sprouts are reported.

Statistical analysis. All experiments were done in triplicate, and duplicate samples from each treatment were analyzed at each sampling time. Mean populations of *S. stanley* in solutions used to soak or wash seeds and sprouts were subjected to analysis of variance and Duncan's multiple range test (SAS Institute, Cary, N.C.) to determine significant differences ($P \leq 0.05$) between treatments.

RESULTS AND DISCUSSION

No salmonellae were detected in uninoculated alfalfa seeds. Populations of *S. stanley* in the two suspensions used to dip seeds were 5.26 and 7.26 log₁₀ CFU/ml and were not changed by dipping seeds for 1 min. The pathogen was not detected (<1 CFU/g) in dried seeds that had been dipped in the small-population suspension; a population of 2.53 log₁₀ CFU/g (339 CFU/g) was detected in seeds dipped in the large-population suspension. At least two factors may have influenced our inability to obtain larger populations of *S. stanley* on the seeds. First, alfalfa seeds are covered with a waxy material that repels

TABLE 1. Populations of *S. stanley* recovered from inoculated alfalfa seeds dipped in chlorine solutions

Initial population (CFU/g of seed)	Chlorine concn (µg/ml) in dip solution	Dip time (min)	Population recovered (mean) ^a	
			CFU/ml of dip solution ^b	CFU/g of seed ^c
339	0 (control)	5	46.7 b	248 b
		10	160 a	387 a
	100	5	0.3 b	128 cd
		10	6.7 b	197 bc
	290	5	1.3 b	59 def
		10	0.7 b	99 de
480	5	5	0 b	48 def
		10	0 b	64 def
	1,010	5	0 b	8 f
		10	0 b	37 ef
65	2,040	5	0	0
		10	0	0
	3,990	5	0	0
		10	0	0

^a Mean values within a column (for 0 to 1,010 µg of chlorine per ml) that are not followed by the same letter are significantly different ($P \leq 0.05$).

^b Average for three 1-ml samples from three replicates. The lower limit of detection was 1 CFU/ml of dip solution.

^c Average for three 25-g samples from three replicates.

water and, thus, *S. stanley* cells suspended in water. Second, it is likely that phenolic compounds present in the seed coat are toxic to a portion of *S. stanley* cells. We have observed that tannins in packing tissue in pecan nuts, for example, are also toxic to salmonellae (4). Some of the cells that adhered to the seeds during the dip process may have been killed during the subsequent drying period and during storage before chlorine and hot water treatments were initiated. We used the seeds containing 339 CFU/g for all experiments except those involving treatments with target concentrations of 2,000 and 4,000 µg of chlorine per ml. A population of 339 CFU/g of seed would be considered large in a commercial setting and, in reality, would be unlikely to occur. If present on alfalfa seeds, populations would more likely be at least 100-fold smaller than that on seeds used in our experiments.

Storage of dry seeds at 8°C for 9 weeks resulted in a reduction in *S. stanley* population from 339 CFU/g to 65 (log₁₀ 1.81) CFU/g. Inactivation was enhanced at 21°C. Storage of dry seeds at 8°C for 1 week and then at 21°C for 8 weeks caused a decrease in population to 8.3 (0.92 log₁₀) CFU/g.

Studies of the use of chlorine dips to kill *S. stanley* on inoculated alfalfa seeds revealed two findings. First, larger populations were recovered from seeds that had been dipped for 10 min than were recovered from seeds that had been dipped for 5 min (Table 1). This difference was statistically significant ($P \leq 0.05$) only for the control (unchlorinated water dip), but it was present at all chlorine dip concentrations. Populations recovered from the dip solutions themselves were also larger for solutions in which seeds had been dipped for 10 min than for those in which they had been dipped for 5 min. Again, the difference was consistent but was statistically significant only for unchlorinated water. These observations imply that with longer dip times, *Salmonella* cells that are initially protected, perhaps in cracks and crevices in the seed coat, become accessible for detection. Therefore, we will focus the analysis on the results of the longer (10 min) dip time. The second important finding is that higher concentrations of chlorine led to greater reductions in *S. stanley* populations on seed but the magnitude of reduction was not directly correlated to concentration. As

TABLE 2. Populations of *S. stanley* on alfalfa seeds dipped in water

Water temp (°C)	Dip time (min)	Population	
		CFU/ml of dip solution ^a	CFU/g of seed ^b
21	5	14	263
	10	14	261
54	5	0.7	9
	10	0.7	6
≥57	5	0	0
	10	0	0

^a Average for three 1-ml samples from three replicates.

^b Average for three 25-g samples from three replicates; dry seeds subjected to treatments contained 339 CFU/g.

shown in Table 1, dipping seeds with an initial population of 339 CFU/g for 10 min in 100- μ g/ml chlorine solution led to significant reductions in population and dipping in 290 μ g of chlorine per ml caused an additional significant reduction in population. Higher concentrations of chlorine (up to 1,010 μ g/ml) in the dip solution caused further reductions in population, although they were not individually statistically significant. Seeds with an initial population of 65 rather than 339 CFU/g were used for experiments with 2,040- and 3,990- μ g/ml chlorine solutions. At these chlorine concentrations, no viable *S. stanley* cells were detected, although enrichment techniques that may have revealed the presence of small numbers of *S. stanley* cells were not employed in these studies.

It is likely that a small percentage of the seeds used in our experiments were in some way damaged. Indeed, 3 to 4% of the untreated seeds were incapable of germinating. Seeds that somehow had been crushed, infected by molds, or damaged by insect infestation or that simply did not develop properly in the field would likely have compromised barriers to infiltration of *S. stanley* during the inoculation procedure. The process of drying seeds after inoculation would, in effect, protect *S. stanley* cells that had entered the seeds through cracks and crevices against inactivation by chlorine. When active chlorine comes in contact with an environment with a high level of organic matter, such as alfalfa seed, potency is quickly diminished. This was very likely happening in our experiments, resulting in failure of even 1,010 μ g of chlorine per ml in the dip solution to inactivate *S. stanley* cells that infiltrated seeds. Since the 0.1% peptone would be expected to neutralize the activity of any chlorine remaining on the seeds after decanting of the treatment solution, it is presumed that the increase in population of *S. stanley* in the peptone wash solution was due to the pummeling action which released *S. stanley* cells embedded in cracks in the seed coat or actually underneath it. The increases in CFU per milliliter of dip solution and CFU per gram of seed appearing in the 10-min treatment compared with the 5-min treatment when the control dip solution (0 μ g of chlorine per ml) was used indicate that longer exposure in peptone solution also facilitates the release of *S. stanley* cells from the seed, in much the same way as the pummeling action does.

Table 2 shows the effect of water temperature on the viability of *S. stanley* on alfalfa seeds. There was no reduction in populations of *S. stanley* on seeds soaked at 21°C for 5 or 10 min (controls). Treatment in water at 54°C reduced *S. stanley* populations from 263 to 9 CFU/g of seed and from 261 to 6 CFU/g of seed after heating for 5 and 10 min, respectively. All other treatment temperatures (57, 60, 63, 66, and 71°C) resulted in elimination (<1 CFU/g) of *S. stanley* from seeds within 5 min.

Results of studies to determine the effect of water temper-

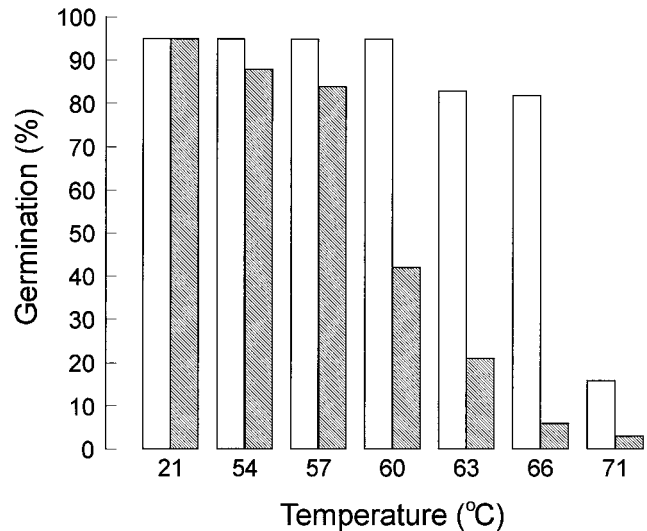


FIG. 1. Effect of dipping alfalfa seeds in hot water for 5 min (□) or 10 min (▨) on germination percentage.

ature on viability of alfalfa seeds are shown in Fig. 1. Compared with dipping seeds in water at 21°C (control), dipping seeds in water at 54, 57, or 60°C for 5 min did not substantially reduce the percentage of seeds that germinated after 48 h at 30°C. However, treatment at 54, 57, and 60°C for 10 min did reduce viability from 96% (control) to 88, 84, and 42%, respectively. Treatment of seeds at 63 and 66°C for 5 min reduced the germination percentage to 83 and 82%, respectively, while treatment at the same temperatures for 10 min reduced viability to 21 and 6%, respectively. While heat treatment appears to be effective in killing *S. stanley* on alfalfa seeds, the range of temperatures that can be used is narrow, i.e., between 57 and 60°C for no longer than 5 min, because lower temperatures may not kill *S. stanley* and perhaps other salmonellae and higher temperatures or a longer exposure time (10 min) decreases germination.

The behavior of *S. stanley* on alfalfa seeds during soaking, germination, sprouting, and refrigerated storage is shown in Fig. 2. An initial population of 3.29 log₁₀ CFU/g of dry seeds increased only slightly during the 6-h soak period, but it in-

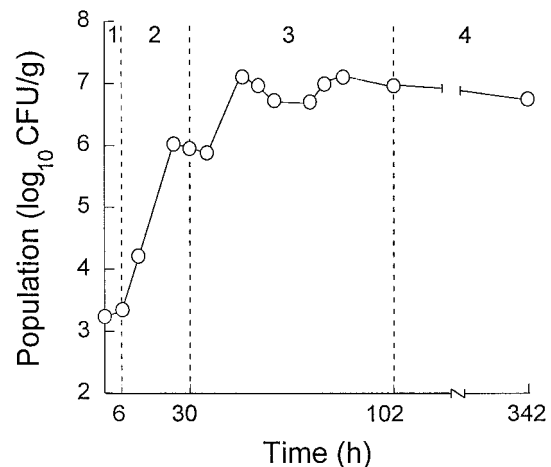


FIG. 2. Growth of *S. stanley* on alfalfa seeds during soaking (1), germination (2), and sprouting (3) and survival during refrigerated storage (4).

creased from 3.32 to 5.97 log₁₀ CFU/g during the 24-h germination step following soaking. During the first 18 h of sprouting, the *S. stanley* population increased to 7.08 log₁₀ CFU/g, and it varied from 6.75 to 7.08 log₁₀ CFU/g through the additional 54 h of sprouting. Populations decreased only slightly during the subsequent 10-day storage period at 5°C.

This study showed that treatment of alfalfa seed contaminated with *S. stanley* using chlorine concentrations of up to 1,040 µg/ml is not effective in eliminating the pathogen, although significant reduction in numbers of viable cells can be achieved. Treatment in hot water is lethal to *S. stanley* but may also decrease germinability of seeds and thus would not be practical in a commercial setting. Treatment of alfalfa seeds with up to 5,000 µg of chlorine per ml does not substantially reduce viability (5). We therefore suggest that a 2,000- to 4,000-µg/ml chlorine soak treatment of alfalfa seeds before germination be used as a method to greatly reduce populations of *S. stanley*, and possibly other salmonellae, while not adversely affecting germination. Nevertheless, because populations of *S. stanley* increased dramatically during the sprouting process, even the 2,000- to 4,000-µg/ml chlorine soak cannot guarantee that sprouts will be free of *Salmonella* cells.

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