Reduction of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in Artificially Contaminated Alfalfa Seeds and Mung Beans by Fumigation with Ammonia

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**ABSTRACT**

Sprouts eaten raw are increasingly perceived as hazardous foods because they have been vehicles in outbreaks of foodborne disease, often involving *Escherichia coli* O157:H7 and *Salmonella* Typhimurium. Although the source of these pathogens has not been established, it is known that the seeds usually are already contaminated at the time sprouting begins. Earlier studies had shown that ammonia was lethal to these same pathogens in manure, so it seemed reasonable to determine whether ammonia was effective against them when associated with seeds to be used for sprouting. Experimentally contaminated 10⁷ to 10⁸ CFU/g and dried seeds, intended for sprouting, were sealed in glass jars in which 180 or 300 mg of ammonia/liter of air space was generated by action of ammonium sulfate and sodium hydroxide. Samples were taken after intervals up to 22 h at 20°C. Destruction of approximately 2 to 3 logs was observed with both bacteria associated with alfalfa seeds, versus 5 to 6 logs with mung beans. Greater kills are apparently associated with lower initial bacterial loads. Germination of these seeds was unaffected by the treatment. It appears that this simple treatment could contribute significantly to the safety of sprout production from alfalfa seeds and mung beans.

Seed sprouts have been implicated as vehicles of transmission in outbreaks of foodborne diseases. Fifteen outbreaks have been reported since 1973, 14 of which occurred after 1989 (17). All reported outbreaks except one were caused by *Escherichia coli* O157:H7 or *Salmonella* spp. These pathogens can reach very high numbers during the sprouting process without changing the appearance of the sprouts (1, 4, 7–9, 15).

Many disinfection methods for seeds have been studied, including immersion in NaOCl, H₂O₂, ethanol, hot water, and ozone (1, 2, 11–16); none has reduced *E. coli* O157:H7 or *Salmonella* spp. by more than 3 log units without reducing the viability of the seeds. Experiments with gamma irradiation (3) have indicated that doses in excess of 1 kGy can eliminate *E. coli* O157:H7 in seeds without reducing the germination rate. A recent study (10) showed that a 2- to 4-log reduction of *E. coli* O157:H7 in or on alfalfa seeds could be obtained by successive treatments with lactic acid (5%) and hypochlorite (20,000 ppm active chlorine) solutions. The study also indicated that the treatments caused damage of a large proportion of the microbial cells with the result that they became unable to grow on selective media. None of the treatments prevented regrowth of surviving *E. coli* O157:H7 during sprouting.

The ability of ammonia gas to significantly reduce *E. coli* O157:H7 and *Salmonella* spp. when they are present in poultry manure (6) suggested application of ammonia to disinfect alfalfa seeds and mung beans, which is the topic of the present study.

**MATERIALS AND METHODS**

*Microbial strains and inoculum preparation.* Molecular constructs of *E. coli* O157:H7 and *Salmonella* Typhimurium that are ampicillin resistant and produce proteins that fluoresce green and blue, respectively, under UV light (5) were used. Colonies formed by the two inoculated strains can be differentiated and counted simultaneously in the presence of green fluorescent protein (GFP) and blue fluorescent protein (BFP) as selective agents. The target organisms were grown to a dense population during 24 h at 37°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) that contained 100 ppm ampicillin and 20 ppm novobiocin, collected by centrifugation, resuspended in sterile normal saline (0.85% sodium chloride), and mixed in a 1/1 ratio. The final bacterial suspension had 10⁸ to 10⁹ CFU/ml of *E. coli* O157:H7 and of *Salmonella* Typhimurium.

*Inoculation of seeds and beans.* Alfalfa seeds and mung beans intended for sprouting were obtained from a local grocery. Two hundred grams each of seeds and beans were immersed in 60 ml of mixed bacterial suspension in a sterile tissue culture flask. The wet inoculated seeds were transferred to a sieved container and then kept for 24 h at room temperature in a laminar flow hood.

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FIGURE 1. Survival of experimentally inoculated (a) E. coli O157:H7 and (b) Salmonella Typhimurium on alfalfa seeds, in the presence of 0, 180, and 300 mg/liter of gaseous ammonia, at room temperature (where error bars are not seen, they are covered by the point estimate data symbol).

FIGURE 2. Survival of experimentally inoculated (a) E. coli O157:H7 and (b) Salmonella Typhimurium on mung beans, in the presence of 0, 180, and 300 mg/liter of gaseous ammonia, at room temperature (where error bars are not seen, they are covered by the point estimate data symbol).

Treatment with ammonia. Petri dishes containing ~10 g of the inoculated seeds were placed on 100-ml beakers turned upside down in 1-liter glass jars, one plate per jar. The levels of ammonia generated in the jars were intended to be 180 and 300 mg per liter of air space, on the following basis. Ammonium sulfate, 0.699 or 1.165 g, was placed in the bottoms of the jars with 1 or 2 g of sodium hydroxide, respectively, to achieve a calculated maximum of 180 or 300 mg/liter air space; 5 ml of sterile distilled and deionized water was added. The jars were quickly sealed tightly with electrical tape and kept at room temperature. Samples were taken at predetermined intervals up to 22 h; once a jar was opened, it was not reused in the experiment.

Detection of surviving target organisms. The treated beans and seeds were taken out of the ammonia jar and put into Whirl-Pak bags containing 30 ml normal saline (0.85% NaCl). The bags were shaken vigorously with a pulsifier (Kalyx Biosciences Inc., Nepean, Canada) for 2 min in order to dislodge the organisms from the seed surfaces. Two replicate samples were taken from each bag and assayed individually. Tenfold serial dilutions in saline were plated in triplicate on brain heart infusion agar with 100 ppm ampicillin and 20 ppm novobiocin. Green colonies (E. coli O157:H7) and blue colonies (Salmonella Typhimurium) were counted under UV light (366 nm) after incubation at 37°C for 24 h.

Effect of treatment on germination. Ammonia-treated (300 mg/liter, 24 h at room temperature) alfalfa seeds (3 g) and mung beans (10 g) were washed with three changes of 200 ml tap water to remove residual ammonia (an alternative procedure could be to neutralize the ammonia with acid). The seeds and beans were placed on moist paper towels in petri dishes at 20°C and visually evaluated for percentage germination.

Statistical analyses. Analysis of variance was used to test for any significant difference in the survivals of the bacteria, as a function of time, for both bacteria at both ammonia concentrations. Regression analyses were performed to determine slope or death rate of bacteria in both ammonia treatments. Tests for parallelism were performed to compare two death rates or survival curves of bacteria. Microsoft Excel software was used to perform all statistical analyses.

RESULTS AND DISCUSSION

The destruction of the target organisms was approximately exponential and resulted in 2- to 3-log reductions in 22 h on alfalfa seeds (Fig. 1) and 3- to 5-log reductions on mung beans (Fig. 2). Analysis of variance showed that the reductions were significant ($P < 0.0005$), compared with the controls, for each combination of bacterium, seed type, and ammonia level. E. coli O157:H7 died more rapidly in the presence of 180 than of 300 mg/liter ammonia ($P < 0.05$), whereas the difference was not significant for Salmonella Typhimurium. Only in one system—at 300 mg/liter ammonia, on alfalfa seeds—did E. coli O157:H7 and Salmonella Typhimurium differ significantly ($P < 0.05$) in ammonia susceptibility. In preliminary experiments with lower initial levels of inoculum, greater proportions of killing were recorded (data not shown). Inasmuch as contamination levels in seeds from outbreaks have been far lower than those used here (14), there is reason to expect greater...
effectiveness of the treatment with lower numbers of pathogens; this is under investigation. The difference in effectiveness on alfalfa seeds and mung beans also needs further study.

There was no observable effect of ammonia treatment on the ability of alfalfa seeds and mung beans to germinate. Some other species of seeds were less tolerant of ammonia than these (data not shown). The results indicate that fumigation with ammonia is an effective chemical treatment for reduction of E. coli O157:H7 and Salmonella in alfalfa seeds and mung beans. The explanation may be that ammonia (molecular weight = 17) is a small, electrically neutral molecule that can be expected to reach and penetrate bacterial cells well. Ammonia fumigation is already in commercial use for other purposes. Because the ammonia is not significantly present in the sprouts, just as the presently recommended hypochlorite is removable by rinsing, ammonia should not be subject to review as a food additive.

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REFERENCES