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Reduction of *Salmonella enterica* on Alfalfa Seeds with Acidic Electrolyzed Oxidizing Water and Enhanced Uptake of Acidic Electrolyzed Oxidizing Water into Seeds by Gas Exchange

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ABSTRACT

Alfalfa sprouts have been implicated in several salmonellosis outbreaks in recent years. The disinfectant effects of acidic electrolyzed oxidizing (EO) water against *Salmonella enterica* both in an aqueous system and on artificially contaminated alfalfa seeds were determined. The optimum ratio of seeds to EO water was determined in order to maximize the antimicrobial effect of EO water. Seeds were combined with EO water at ratios (wt/vol) of 1:4, 1:10, 1:20, 1:40, and 1:100, and the characteristics of EO water (pH, oxidation reduction potential [ORP], and free chlorine concentration) were determined. When the ratio of seeds to EO water was increased from 1:4 to 1:100, the pH decreased from 3.82 to 2.63, while the ORP increased from +455 to +1,073 mV. EO water (with a pH of 2.54 to 2.38 and an ORP of +1,083 to +1,092 mV) exhibited strong potential for the inactivation of *S. enterica* in an aqueous system (producing a reduction of at least 6.6 log CFU/ml). Treatment of artificially contaminated alfalfa seeds with EO water at a seed–to–EO water ratio of 1:100 for 15 and 60 min significantly reduced *Salmonella* populations by 2.04 and 1.96 log CFU/g, respectively ($P < 0.05$), while a Butterfield's buffer wash decreased *Salmonella* populations by 0.18 and 0.23 log CFU/g, respectively. After treatment, EO water was *Salmonella* negative by enrichment with or without neutralization. Germination of seeds was not significantly affected ($P > 0.05$) by treatment for up to 60 min in electrolyzed water. The uptake of liquid into the seeds was influenced by the internal gas composition (air, N_2, or O_2) of seeds before the liquid was added.

Sprouted seeds have been associated with several foodborne pathogens, such as *Salmonella, Escherichia coli* O157:H7, and *Bacillus cereus (14, 20, 21).* Vegetable sprouts are perceived as a healthy food and are consumed raw with minimal processing. The presence of *Salmonella* and *E. coli* O157:H7 in seeds and the ability of these pathogens to multiply during growing and harvesting have raised food safety concerns for consumers looking to include sprouts as a healthy choice in their diet. Several salmonellosis outbreaks involving different *Salmonella* serovars have been linked to the consumption of alfalfa sprouts *(14, 20).* Seeds have been reported to be the major source of contamination, although the presence of pathogens may also be due to contaminated water or mishandling during production and distribution *(14, 18).*

Several treatments for the elimination of *Salmonella* from seeds and sprouts have been investigated. Chlorine *(2, 3, 8, 19),* alkali *(17),* ammonia *(6),* hydrogen peroxide *(1),* several natural volatile components *(25),* and gamma radiation *(16),* as well as chemical treatments combined with heat *(8, 19)* or ultrasound *(17),* have been evaluated for their ability to reduce or eliminate the pathogen. With the exception of gamma radiation, all of these treatments have failed to completely eliminate the pathogen. None of the chemical treatments alone reduced *Salmonella* populations on alfalfa seeds or sprouts by >3.5 log CFU/g without dramatically affecting the viability of seeds or the sensory characteristics of sprouts. In this study, we report the use of electrolyzed water for alfalfa seed disinfection.

Electrolysis of a NaCl solution has long been used for chlorine production. Electrolyzed water is produced through the electrolysis of a dilute solution of NaCl with the use of an instrument in which the anode and cathode are separated by a membrane. Electrolyzed water has been used for several years in Japan for disinfecting medical instruments. In recent years, electrolyzed water as a disinfectant has generated interest in the food industry. In suspension tests, the water collected at the anode has been shown to have strong bactericidal effects on *E. coli* O157:H7, *Listeria monocytogenes, Salmonella* Enteritidis, *Campylobacter jejuni,* and *B. cereus (15, 23).* This new disinfecting agent has also been tested for the disinfection of kitchen cutting boards and other surfaces *(15, 22)* as well as lettuce, fresh-cut vegetables *(7, 11),* and, very recently, alfalfa seeds *(10).* The electrolysis of water containing sodium chloride imparts a strong oxidizing character to the water collected at the anode, which has potential as a seed disinfection agent.

Disinfection of seeds used for sprouting is difficult, since the seeds may present surface irregularities or physical damage that may protect bacteria from the action of water-based disinfectants. A mechanism for enhancing the uptake of the antimicrobial agent into the outer layers of the seed might improve the disinfection step.

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TABLE 1. *Bactericidal effects of a 15-min EO water (HEW, SOW) treatment against a four-strain* Salmonella *mixture in an aqueous system*[a]

Type of EO water	pH	ORP (mV)	*Salmonella* population (log CFU/ml)			
			Initially		After EO water treatment	
			TSAN	BSAN	TSAN	BSAN
HEW	2.54	+1,083	6.68 ± 0.04	6.53 ± 0.01	0[b]	0[b]
SOW	2.38	+1,092	6.68 ± 0.04	6.53 ± 0.01	0[b]	0[b]

[a] ORP, oxidation reduction potential; HEW, EO water obtained with the Hoshizaki electrolysis unit; SOW, EO water obtained with the Super Water Mini electrolysis unit. The values shown are means ± standard deviations for two duplicate experiments.
[b] Negative by enrichment in TSBN and RVBN with or without neutralization; no detectable survivors by direct plating.

In previous studies *(4, 5),* exchanging the internal gas of fresh cucumbers with oxygen has been found to result in a fully cured appearance for the brined cucumbers within 1 or 2 days, as compared with several months for nonexchanged cucumbers. The metabolism of oxygen to carbon dioxide in the oxygen-exchanged cucumbers, along with the subsequent diffusion of carbon dioxide into the tissue fluid (to a greater extent than that of oxygen), resulted in a vacuum within the tissue that drew brine into the cucumbers.

We decided to investigate whether exchanging the internal gas of seeds with O_2 results in better absorption of the electrolyzed water into the seeds and a subsequent improvement in disinfection.

The objectives of this study were (i) to determine the efficacy of acidic electrolyzed oxidizing (EO) water in eliminating *Salmonella enterica* serovars Stanley, Typhimurium, Hartford, and Mbandaka both in an aqueous system and on artificially contaminated alfalfa seeds; (ii) to determine the optimum ratio of seeds to EO water in order to maximize the antimicrobial effect of EO water; and (iii) to determine whether exchanging the internal gas of seeds with oxygen results in an improved uptake of liquid into alfalfa seeds and the subsequent effect on seed disinfection.

MATERIALS AND METHODS

Bacterial strains. Four *Salmonella* serovars were used in this study: *Salmonella enterica* Stanley (from an alfalfa sprout–associated outbreak, obtained from Dr. William Fett, Eastern Regional Research Center, U.S. Department of Agriculture), *Salmonella enterica* Mbandaka (from an alfalfa sprout–associated outbreak, obtained from the Oregon Public Health Laboratory, Salem, Oreg.), *Salmonella enterica* Typhimurium ATCC 14028, and *Salmonella* Hartford (from an orange juice–associated outbreak, obtained from Dr. M. E. Parish, University of Florida, Lake Alfred, Fla.). Cells were adapted to grow in tryptic soy broth (Difco Laboratories, Detroit, Mich.) supplemented with 50 μg of nalidixic acid (Sigma Chemical Co., St. Louis, Mo.) per ml (TSBN).

Contamination of alfalfa seeds. The four *Salmonella* serovars were grown in 100 ml of TSBN at 37°C for 24 h. The cultures were centrifuged at 8,000 rpm for 10 min at 21°C, and the pellets were resuspended in 250 ml of Butterfield's phosphate buffer (Hardy Diagnostics, Santa Maria, Calif.). The suspension was centrifuged again at 8,000 rpm for 5 min at 21°C, and the pellet was resuspended in 250 ml of Butterfield's buffer. Alfalfa seeds (Lucerne, Australian) were purchased from International Specialty Supply (Cookeville, Tenn.). Seeds (250 g) were rinsed twice with 250 ml of sterile distilled water and then gently mixed with the four-strain suspension for 1 min. The suspension was decanted and the seeds were placed in a layer of ca. 7 mm on an aluminum screen lined with a double layer of cheesecloth. Seeds were air dried under a biosafety class II hood for 7 h at room temperature (22 ± 1°C). After 5 h, the seeds were visibly dry. Dry seeds (10 g) were placed in Ziploc bags (Hefty, Pactiv, Ill.), stored at 4 to 5°C, and used within 15 days.

Preparation of electrolyzed water. Two types of electrolyzed water generators were used in this study. EO (HEW) water was prepared with a Hoshizaki ROX 20TA-U continuous generator (Hoshizaki Electric Co. Ltd., Japan) at 10 V and 14.0 ± 0.4 A. Deionized water and a 13.6% (wt/vol) NaCl solution were simultaneously pumped into the generator. EO (SOW) water was prepared with a batch type JED-007 Super Water Mini generator (Altex Janix, Kanagawa, Japan) and a 0.05% (wt/vol) NaCl solution; a 10-min electrolysis time was used. Electrolyzed water was used within 1 h after production. The characteristics of EO water, including pH, oxidation reduction potential (ORP), and free chlorine concentration, were determined. The pH and ORP were determined with a digital ion analyzer (Orion Research Inc., Beverly, Mass.) equipped with pH and ORP electrodes (ACCUMET, Denver Instrument Co, Denver, Colo.). Free chlorine concentrations were measured by the DPD (*N,N*-diethyl-*p*-phenylenediamine) colorimetric method with a Pocket Colorimeter (Hach Company, Loveland, Colo.).

Bacterial analysis for aqueous and contaminated seed treatments. A four-strain *Salmonella* mixture was obtained by combining equal volumes of the four *Salmonella* serovars grown in TSBN for 24 h. For the aqueous system, treatments were carried out by combining 1 ml of the *Salmonella* mixture with 100 ml of electrolyzed water for 15 min at room temperature (22°C). The treatment solution was surface plated on bismuth sulfite agar (Difco) supplemented with 50 μg of nalidixic acid per ml (BSAN) and on tryptic soy agar (Difco) supplemented with 50 μg of nalidixic acid per ml (TSAN) and incubated at 37°C for 24 h. Enrichment of the treated sample was carried out without neutralization by combining 1 ml of treatment solution with 9 ml of TSBN or Rappaport-Vassiliadis broth (RVBN) and with neutralization by neutralizing 1 ml of the treatment solution with 2.5 ml of neutralizing buffer and then adding 9 ml of TSBN or RVBN. TSBN and RVBN tubes were incubated at 37 and 42°C, respectively, for 48 h.

Contaminated alfalfa seeds (10 g) were treated with 1 liter of EO water or Butterfield's buffer (control) for 15 and 60 min on a rotary shaker (Environ shaker, Lab-Line, Melrose Park, Ill.) at 150 rpm. The treatments were carried out at room temperature (23.5 ± 2°C). The treatment solution was poured, and the seeds

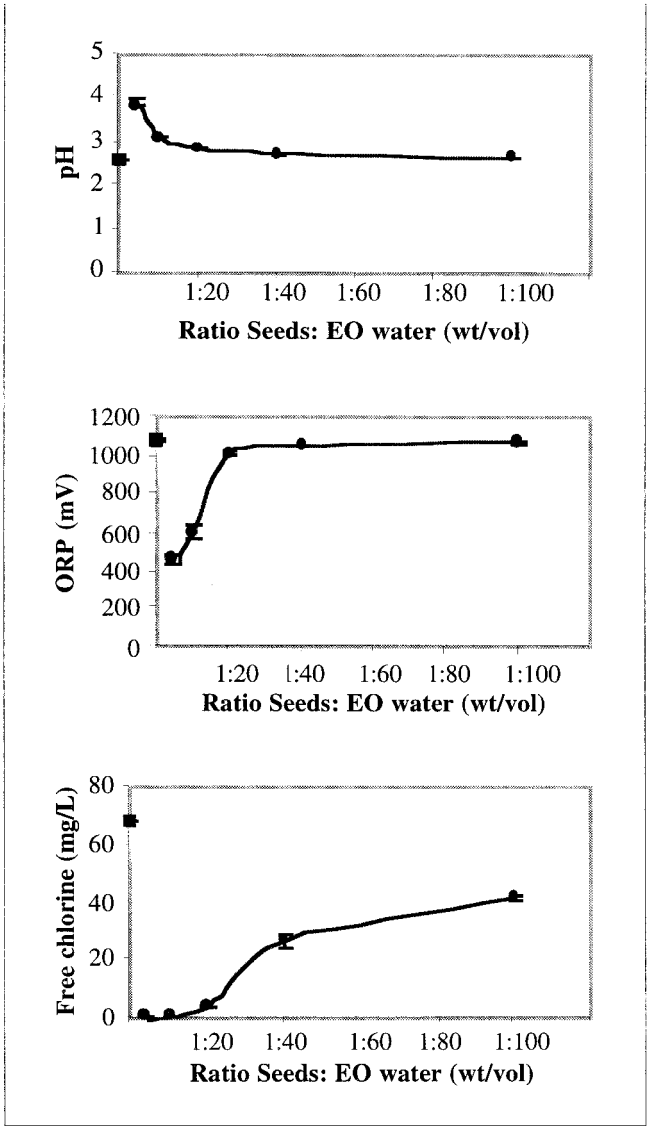

FIGURE 1. *Influence of EO water volume on pH, ORP, and free chlorine concentration. Vertical bars indicate standard deviations from the mean. Seeds were combined with EO water at ratios (wt/vol) of 1:4, 1:10, 1:20, 1:40, and 1:100 and agitated for 15 min at 120 rpm. The pH, ORP, and free chlorine concentration of EO water before treatment are shown as dark squares.*

were mixed with 90 ml of neutralizing buffer (Difco) and transferred to stomacher bags. After the seeds had been pummeled for 1 min at 230 rpm in a stomacher blender (Stomacher 400 Circulator, Seward, London, UK), the neutralizing buffer wash was surface plated on BSAN with a spiral plater (Autoplate 4000, Spiral Biotech, Inc., Norwood, Mass.). After the plates had been incubated at 37°C for 24 h, randomly selected colonies having morphological characteristics typical of *Salmonella* were confirmed with the use of triple sugar iron agar (Difco) and the API 20E diagnostic kit (bioMérieux Vitek, Inc., Hazelwood, Mo.).

Seed germination. Control and treated seeds were tested for germination potential. Seeds were rinsed two times with 100 ml of sterile deionized water to remove the residual electrolyzed water. Approximately 100 seeds were spread between two moistened paper towels, which were rolled and placed in a plastic container. The seeds were incubated at 15°C for 7 days. Each seed was examined for the development of a radicle, and the percentage of seeds that germinated was calculated.

Determination of the effects of seed–to–EO water ratio on the properties of EO water. Seeds (10 g) were combined with EO water at ratios (wt/vol) of 1:4, 1:10, 1:20, 1:40, and 1:100 and agitated at 22°C on a platform shaker for 15 min at 120 rpm. After the 15-min treatment, the characteristics of EO water (pH, ORP, and free chlorine concentration) were determined as described above.

Gas exchange of alfalfa seeds and bacterial analysis. Alfalfa seeds were obtained from International Specialty Supply and had a Canadian provenience (Canada lot no. NS9-188). Alfalfa seeds (400 g) were placed into a 2-liter Erlenmeyer flask fitted with inlets for gas and water with a graduated water reservoir to monitor liquid uptake into seeds. Seeds were exposed to either oxygen or nitrogen at a flow rate of 1.5 liters/min for 1 h to exchange the internal atmosphere. A control was represented by nonexchanged (air) seeds. After the gas exchange, EO water (HEW and SOW) and distilled water (control) were added to the flasks while a constant gas pressure was maintained to prevent air from entering the flask. The liquid uptake was monitored at 20°C for 150 min.

Contaminated alfalfa seeds (10 g) were gas exchanged with oxygen and air (control) by the procedure described above. Seeds were treated with EO (HEW) water for 150 min, and the population of *Salmonella* was determined by mixing the seeds with 90 ml of Butterfield's buffer in a stomacher for 1 min at 230 rpm and then surface plating the suspension on BSAN.

Statistical analysis. Statistical analysis was performed with S-PLUS 2000 software. All experiments were replicated at least once. Reported plate counts represent mean values ± standard deviations obtained from two or three replicate experiments. Data

TABLE 2. *Bactericidal effects of EO (HEW) water applied at a ratio of 1 part seed to 100 parts EO water against a four-strain* Salmonella *mixture inoculated onto alfalfa seed*[a]

Treatment time (min)	pH	ORP (mV)	Free chlorine concn (mg/liter)	*Salmonella* population (log CFU/g)[b] Initially	After control	After EO water treatment
15	2.50	+1,079	70.3	5.29 ± 0.04 A	5.11 ± 0.19	3.25 ± 0.08 B
60	2.67	+1,076	66.8	5.15 ± 0.05 A	4.92 ± 0.26	3.19 ± 0.14 B

[a] HEW, EO water obtained with the Hoshizaki electrolysis unit; ORP, oxidation reduction potential.

[b] Mean ± standard deviation for two or three duplicate experiments. Means with different letters in the same row are significantly different ($P < 0.05$).

TABLE 3. *Effects of EO (HEW) water treatment on seed germination*[a]

Treatment time (min)	Parameters for tap water			Parameters for EO (HEW) water		
	ORP (mV)	pH	Germination %	ORP (mV)	pH	Germination %
15	+692	6.21	95.2 ± 1.5 A	+1,079	2.50	95.4 ± 1.0 A
60	+744	6.27	94.1 ± 0.6 A	+1,074	2.58	93.8 ± 1.6 A

[a] HEW, EO water obtained with the Hoshizaki electrolysis unit; ORP, oxidation reduction potential. Germination percentages are presented as means ± standard deviations for two triplicate experiments. Means with the same letter in the same row are not significantly different ($P > 0.05$).

were analyzed by a fixed-effects analysis of variance with a 95% confidence level. Bacterial counts were logarithmically transformed prior to statistical analysis.

RESULTS AND DISCUSSION

Efficacy of EO water in killing *Salmonella* in an aqueous system. The inactivation of the four-strain *Salmonella* mixture in an aqueous system and the properties (pH and ORP) of electrolyzed water are presented in Table 1. The population of *Salmonella* decreased to undetectable levels after a 15-min treatment in an aqueous system, indicating that a *Salmonella* reduction of at least 6.6 log CFU/ml was achieved with electrolyzed water. These results are consistent with earlier findings indicating that electrolyzed water was very effective in eliminating *Salmonella* Enteritidis in an aqueous system within 10 min *(23).* We confirmed that electrolyzed water was effective against a mixture of four *Salmonella enterica* serovars in an aqueous system.

Effect of seed–to–EO water ratio on the characteristics of EO water. The characteristics of the electrolyzed water after treatment at different ratios of seed to EO water are presented in Figure 1. Seeds were combined with EO water at ratios (wt/vol) of 1:4, 1:10, 1:20, 1:40, and 1:100, and the characteristics of EO water (pH, ORP, and free chlorine concentration) were determined. When the ratio of seeds to EO water was increased from 1:4 to 1:100, the pH decreased from 3.82 to 2.63 and the ORP increased from +455 to +1,073 mV.

Efficacy of EO water in killing *Salmonella* on contaminated alfalfa seeds. Populations of *Salmonella* recovered from alfalfa seeds after treatment with electrolyzed water for 15 and 60 min and the characteristics of EO water (pH, ORP, and free chlorine concentration) prior to the addition of seeds are listed in Table 2. The treatments were carried out for 15 and 60 min to determine the effect of treatment time on seed disinfection. An increase in the treatment time did not result in an increase in the reduction of bacteria. EO water significantly reduced *Salmonella* levels on alfalfa seeds by 2.04 and 1.96 log CFU/g within 15 and 60 min, respectively ($P < 0.05$). No viable cells were recovered from the electrolyzed water treatment solution with or without neutralization.

Effect of EO water on seed germination. The effect of electrolyzed water on seed germination was determined in order to assess the applicability of EO water for seed disinfection. Germination percentages for seeds treated with electrolyzed water for 15 and 60 min were similar to those for seeds washed with tap water (control seeds) (Table 3). Hence, it was shown that a EO water treatment for up to 60 min did not significantly affect seed germination ($P > 0.05$).

Effects of oxygen-exchanged seeds on the uptake of electrolyzed water and the subsequent effects on seed disinfection. The uptake of EO water and distilled water (control) by gas-exchanged alfalfa seeds is presented in Figures 2 through 4. Liquid uptake was influenced by the internal gas composition of the seeds before liquid was added. For oxygen exchanged seeds, the higher uptake of liquid

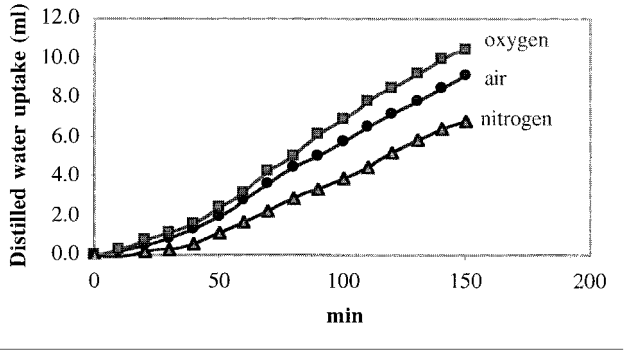

FIGURE 2. *Distilled-water uptake by gas-exchanged alfalfa seeds. Seeds (400 g) were exposed to oxygen and nitrogen at a flow rate of 1.5 liters/min for 1 h before distilled water was added to the flask while a constant gas pressure was maintained to prevent air from entering the flask.*

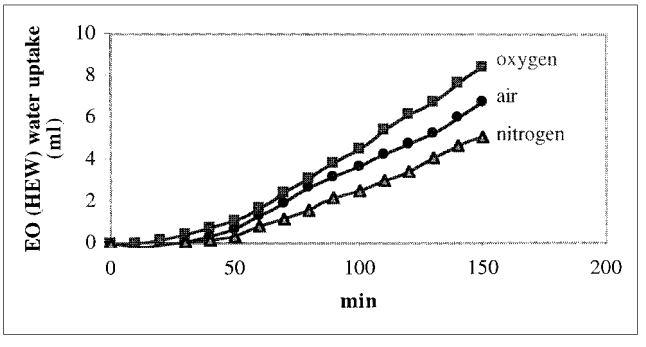

FIGURE 3. *EO (HEW) water uptake by gas-exchanged alfalfa seeds. Seeds (400 g) were exposed to oxygen and nitrogen at a flow rate of 1.5 liters/min for 1 h before EO (HEW) water was added to the flask while a constant gas pressure was maintained to prevent air from entering the flask.*

TABLE 4. *Effects of treatment with EO (HEW) water on a four-strain* Salmonella *mixture in gas-exchanged alfalfa seeds*[a]

Treatment	pH	ORP (mV)	*Salmonella* population (log CFU/g)[b] Initially	After EO water treatment
Air	2.56	+1,101	6.60 ± 0.29 A	4.63 ± 0.21 B
Oxygen	2.56	+1,101	6.60 ± 0.29 A	4.33 ± 0.13 B

[a] HEW, EO water obtained with the Hoshizaki electrolysis unit.
[b] Mean ± standard deviation for two duplicate experiments. Means with different letters in the same row are significantly different ($P < 0.05$).

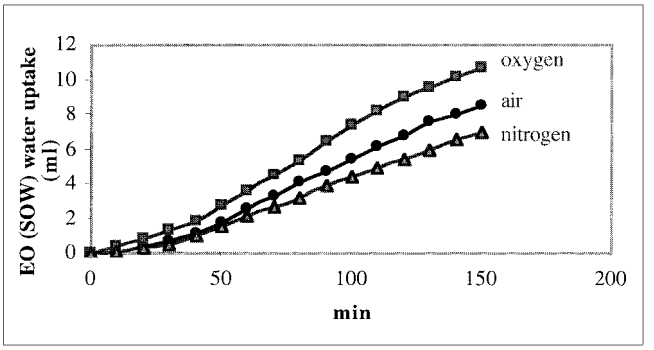

FIGURE 4. *EO (SOW) water uptake by gas-exchanged alfalfa seeds. Seeds (400 g) were exposed to oxygen and nitrogen at a flow rate of 1.5 liters/min for 1 h before EO (SOW) water was added to the flask while a constant gas pressure was maintained to prevent air from entering the flask.*

was probably induced by the vacuum created within the seeds as a consequence of O_2 depletion during metabolism. However, the uptake of liquid in the nitrogen-exchanged seeds showed that diffusion processes are responsible for the largest part of the uptake. The effect of the oxygen exchange procedure on bacterial inactivation is presented in Table 4. The populations of *Salmonella* in the air- and oxygen-exchanged seeds were significantly reduced by 1.97 and 2.27 log CFU/g, respectively ($P < 0.05$). The small difference between reductions achieved for air- and oxygen-exchanged seeds might be due to an overall low uptake of liquid into the seed combined with rapid inactivation of electrolyzed water inside the seed. Germination of seeds was not significantly affected by the oxygen exchange procedure ($P > 0.05$) (Table 5).

Recently, it was reported that the treatment of seeds with electrolyzed water at a seed–to–electrolyzed water ratio of 1:9 resulted in a bacterial reduction of 1.5 log CFU/g, with the reduction increasing to 3.7 log CFU/g when the electrolyzed water treatment was combined with sonication and seed coat removal *(10).*

In the present study, the efficacy of EO water in killing *Salmonella* both in an aqueous system and on contaminated alfalfa seeds was determined. An increase in the treatment time did not result in an increase in bacterial reduction on contaminated seeds. This finding might be due to a reduction in the antimicrobial characteristics of electrolyzed water with an increase in the time of contact with organic material from the seeds. Electrolysis of a dilute solution of NaCl generated an aggregate of antimicrobial factors, including low pH, chlorine, and high ORP. It has been suggested that electrolyzed water contains, in addition to available chlorine, various reactive oxygen species such as ·O, ·OH *(11),* and O_3. These reactive oxygen species are known as strong oxidants and would therefore have strong antimicrobial effects. EO water had a pH of 2.38 to 2.67. At a low pH, HOCl is undissociated and is more effective than the hypochlorite ion (OCl^-). The radical ·OH could come both from the dissociation of HOCl and from the decomposition of ozone in an aqueous system. It was expected that the electrolyzed water would be less effective in a system rich in organic matter (e.g., seeds) than in an aqueous system, since the free chlorine and the radical species are rapidly inactivated by contact with organic material.

Disinfection of seeds prior to sprouting is critical in obtaining a safe product. Results of studies involving alfalfa seeds inoculated with low levels of *Salmonella* have suggested that the number of organisms present on seeds may increase up to 10,000-fold during the sprouting process, with *Salmonella* reaching populations of up to 10^7 CFU/g on alfalfa sprouts *(8).* Although several seed disinfection treatments have been proposed, these methods have proved to be unsatisfactory. Surface irregularities, physical damage (e.g., cracks, wrinkles, missing testa parts) to seeds, and the presence of microbial biofilms on seed and sprout surfaces *(24)* may compromise the efficacy of disinfectants. The presence of cracks and openings in the seed coat may facilitate the entry of bacteria into the seed and will therefore enhance the survival of pathogens inside the seed. Bacteria can also enter through the vascular system and through the hilum of the ripened seed *(13).* Once pathogens are local-

TABLE 5. *Effects of the gas exchange procedure on seed germination*[a]

Exchange gas	Parameters for distilled water pH	ORP (mV)	Germination %	Parameters for EO (HEW) water pH	ORP (mV)	Germination %	Parameters for EO (SOW) water pH	ORP (mV)	Germination %
Air	3.98	+407	70.5 ± 4.0 A	2.57	+1,109	66.6 ± 3.4 A	2.52	+1,108	59.6 ± 5.8 A
Oxygen	3.98	+407	70.1 ± 4.4 A	2.57	+1,109	66.7 ± 2.6 A	2.52	+1,108	64.0 ± 6.9 A
Nitrogen	3.98	+407	65.9 ± 3.3	2.57	+1,109	63.6 ± 2.5	2.52	+1,108	63.3 ± 4.7

[a] HEW, EO water obtained with the Hoshizaki electrolysis unit; SOW, EO water obtained with the Super Water Mini electrolysis unit. Germination percentages are presented as means ± standard deviations for two triplicate experiments. Means with the same letter in the same column are not significantly different ($P > 0.05$).

ized inside the seed, the disinfection of seeds will be difficult. The effectiveness of EO water as a disinfectant agent will depend on its ability to make contact with the target microorganism and its ratio to organic material.

To date, the antimicrobial mechanism of EO water has not been fully understood. The concentration of available chlorine *(11, 12)* and the high ORP values *(9)* have been reported to contribute to the bactericidal properties of EO water. EO water might provide an effective means of disinfecting alfalfa seeds, but further investigations must be performed to prevent the rapid decrease in the antimicrobial effect of electrolyzed water in contact with organic material.

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