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Effect of sequential dry heat and hydrogen peroxide treatment on inactivation of *Salmonella* Typhimurium on alfalfa seeds and seeds germination

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ABSTRACT

The purpose of this study was to inactivate *Salmonella* Typhimurium on alfalfa seeds without having negative effect on seed germination. Inoculated alfalfa seeds were treated with dry heat at 60, 70 or 80 °C for 0, 12, 18 or 24 h followed by 2% hydrogen peroxide solution (10 min). Populations of *Salmonella* on alfalfa seeds treated with dry heat alone (60, 70 or 80 °C) for up to 24 h were reduced by 0.26 −2.76 log CFU/g, and sequential treatment with dry heat and H_2O_2 reduced populations by 1.66 −3.60 log CFU/g. The germination percentage of seeds subjected to sequential treatments was significantly enhanced to up to 97%, whereas that of untreated seeds was only 79.5%. This study suggests that sequential treatment with dry heat and hydrogen peroxide is applicable for reducing levels of *Salmonella* on seeds while simultaneously enhancing seeds germinability.

1. Introduction

As many consumers worldwide have shifted their dietary choices to include healthful foods, sprouts are being highlighted because of their nutritive value. Alfalfa sprouts are one of the more popular forms of spouts consumed around the world, and their consumption has increased in the last few decades. Unlike in Asia, a considerable amount of alfalfa sprouts is consumed raw in Europe and the United States. Raw sprouts can carry foodborne pathogens, and can be implicated in foodborne illnesses. A number of outbreaks associated with consumption of raw alfalfa sprouts have been reported, and many of them involved *Salmonella* spp. (Taormina et al., 1999). In 1995 large outbreaks of *Salmonella* Stanley, linked to alfalfa sprouts, occurred in Finland and 17 states of the USA (Mahon et al., 1997). In 2009, simultaneous outbreak of *Salmonella* Saintpaul associated with alfalfa sprouts were reported from 14 states in the USA, resulting in 235 infections (CDC, 2009).

Potential sources for contaminating sprouts with pathogenic bacteria are various, including seeds, contaminated irrigation water, inadequately treated animal manure, poor sanitation equipment, and mishandling by growers. Judging from previous outbreaks and epidemiological investigations, seeds are the most likely source of sprout-associated outbreaks (NACMCF, 1999a). Pathogens trapped in or on seeds proliferate exponentially during sprouting (NACMCF, 1999b), resulting in a high risk of illness, since the warm and humid environment during sprouting provides perfect conditions needed for pathogens to multiply rapidly.

A number of decontamination studies, such as ones involving heat treatment (Bari et al., 2008; Enomoto et al., 2002; Hu et al., 2004), sanitizers (Taormina and Beuchat, 1998; Beuchat et al., 2001; Soylemez et al., 2001; Beuchat and Scouten, 2002), organic acids (Lang et al., 2000), ozonated water (Sharma et al., 2001; Wade el al., 2003), electrolyzed oxidizing water (Kim et al., 2003), and UV irradiation (Sharma and Demirci, 2003) have been conducted to develop interventions for reducing the *Salmonella* load on alfalfa seeds. However, no treatment can guarantee complete elimination of pathogens. The U.S. Food and Drug Administration (FDA) and National Advisory Committee on Microbiological Criteria for Foods (NACMCF) recommended that seeds be sanitized with 20,000 ppm of calcium hypochlorite prior to sprouting. Nevertheless, calcium hypochlorite can corrode metal equipment, and can potentially

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produce toxic fumes, and also requires strict precaution in handling. Hydrogen peroxide is a GRAS (Generally recognized as safe) compound, and its germicidal effect has been widely evaluated in recent years. Its activity involves in formation of hydroxyl free radicals (•OH), which act as an oxidizers of bacteria cell components (McDonnell and Russell, 1999).

Thermal treatment effectively eliminates pathogens on fresh produce, including seeds, and it has been studied for a long time (Jaquette et al., 1996; Scouten and Beuchat, 2002). Dry heat, which is one of thermal methods, has been shown to be successful alone or when combined with other treatments for seed decontamination (Bari et al., 2009; Neetoo and Chen, 2011). It has achieved a certain degree of reduction while affecting the seed germination rate less than other interventions. While hydrogen peroxide and dry heat have been studied previously for disinfection of alfalfa seeds, they have not been used together.

The aim of this study was to investigate the efficacy of sequential treatments of dry heat and hydrogen peroxide for eliminating *Salmonella enterica* Serovar Typhimurium on alfalfa seeds, and demonstrate the impact of this sequential treatment on seed viability. Specifically, we determined the optimum dry heating time, temperature, and hydrogen peroxide treatment combination to maximize the reduction of *S*. Typhimurium.

2. Materials and methods

2.1. Bacterial strains and preparation of inoculum

Three strains of *S. enterica* subsp. *enteric* serovar Typhimurium (ATCC 19585, ATCC 43971, and DT 104) obtained from the School of Food Science bacteria culture collection of Seoul National University (Seoul, Korea) were used for inoculation. Stock cultures were prepared by growing strains in 5 ml of tryptic soy broth (TSB; Difco, BD) at 37 °C for 24 h, combining 0.7 ml of culture with 0.3 ml of sterile 50% glycerol and then storing at −80 °C. Active cultures were generated by streaking stock cultures onto tryptic soy agar (TSA; Difco, BD), incubating at 37 °C for 24 h and storing at 4 °C for up to one mo. A single colony of each strain was transferred to 5 ml of TSB and incubated at 37 °C for 24 h. One ml of overnight (24 h) culture of each strain was spread onto three TSA plates to produce a bacterial lawn, which was followed by incubation at 37 °C for 24 h. Eight to 9 ml of 0.2% peptone water was added to each plate to harvest the bacterial lawn, and cell suspensions were made by rubbing the agar surface with a sterile swab (3M pipette swab, 3M Korea Ltd.) to dislodge cells. Cell suspensions (ca. 10^{12} CFU/ml) were collected, combined, and augmented with 420 ml of 0.2% peptone water to yield 500 ml of culture cocktail in total.

2.2. Inoculation of culture cocktail on alfalfa seeds

Alfalfa seeds were purchased from a local seed retail company (Danong, Gyeonggi province, Korea Rep.). Approximately 500 g of alfalfa seeds were immersed in 500 ml of *Salmonella* Typhimurium suspension, and gently agitated in this mixture for 5 min. The cell suspension was drained completely, and the seeds were placed onto aluminum foil and dried in a laminar flow hood at 21 °C for 20 h. The dried seeds were sealed in sterile resealable bag and stored in a 4 °C refrigerator for no more than one week.

2.3. Determination of moisture content and water activity changes for drying inoculated alfalfa seeds

Uninoculated alfalfa seeds (500 g) were immersed in 500 ml of peptone water and stirred occasionally for 5 min. Drained seeds were transferred to aluminum foil and dried in a laminar flow hood at 21 °C for 20 h. Moisture content (dry basis) and water activity (a_w) was measured every two hours using a halogen moisture analyzer (HB43-S; Mettler Toledo, Columbus, OH) and a water activity meter (AQUA Lab, Pullman, WA.), respectively.

2.4. Treatments

2.4.1. Dry heat treatment

Approximately 90 g of alfalfa seeds were divided into 10 g portions and placed onto sterile tray-shaped pieces of aluminum foil (70 mm wide by 110 mm long). Seeds were laid less than 5 mm high. These aluminum foil plates were transferred to an oven (OV-11, JEIO Tech, Seoul, Korea Rep.) with the temperature set at 60, 70 or 80 °C. Alfalfa seeds were then heated for 0, 12, 18 or 24 h.

2.4.2. Hydrogen peroxide solution preparation and treatments

Aqueous solutions containing 2% (v/v) hydrogen peroxide (30%, Junsei Cheminal Co. Ltd., Tokyo, Japan) were prepared by adding sterile distilled water (DW). The diluted solutions were used within 20 min. Distilled water served as a control.

Hydrogen peroxide (H_2O_2) solution treatments was conducted on both dry heated and non-heated seed samples. Three aluminum foil plates were removed from the oven at selected time intervals (12, 18, or 24 h). For each time interval, one of the 10 g portions of alfalfa seed served as a control, and one of the other portions was soaked in 2% H_2O_2 solution for 10 min. To determine that inactivation following H_2O_2 exposure was not due to any washing effect, the last 10 g portion was soaked in distilled water for 10 min.

2.5. Microbiological analysis

Each treated seed sample was immediately transferred to a sterile filter stomacher bag (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 90 ml of Dey- Engley (DE) broth (Difco, Becton Dickinson, Sparks, MD, USA), and homogenized for 2 min with a stomacher (EASY MIX, AES Chemunex, Rennes, France). After homogenization, 1 ml aliquots of DE broth-sample mixture were 10-fold serially diluted in 9 ml of 0.2% peptone water. One tenth ml of selected dilutions was spread-plated onto duplicate plates of Xylose Lysine Desoxycholate agar (XLD; Oxoid), a selective medium for enumerating *S*. Typhimurium cells. When low numbers of surviving cells were anticipated, 0.25 ml of undiluted stomacher bag contents was spread-plated onto each of four plates. All XLD plates were incubated at 37 °C for 24 h and characteristic black colonies were counted. Representative colonies were randomly selected and subjected to the *Salmonella* latex agglutination assay (Oxoid, Ogdensberg, NY) to conform their identity.

2.6. Effect of dry heat and hydrogen peroxide on viability of alfalfa seeds

Two hundred randomly selected treated or control seeds were placed on sterile cheesecloth in 90 mm diameter petri dishes, and periodically provided with sufficient distilled water to foster seed germination. The seeds were incubated at room temperature (21 ± 2 °C) for 5 days. Only seeds with a hypocotyl protruding were counted as a sprout. Ruptured or swollen seeds were not counted. Experiments were performed in triplicate.

2.7. Hydrogen peroxide residue evaluation

The level of hydrogen peroxide on alfalfa seeds was monitored by using water quality test strips (Water Works™, Industrial Test System, Inc., USA). This test strip detect a minimum hydrogen peroxide concentration of 0.05 ppm and a maximum of 4.0 ppm

Hydrogen peroxide – roughly detect 0.05, 0.3, 0.5, 1.0, 2.0, 4.0 ppm respectively. Treated alfalfa seeds were assayed for H_2O_2 at various time intervals throughout 7 days of incubation on the assumption that consumers eat sprouts at different stages of sprout development. Ten grams of sprouts were transferred to a sterile stomacher strainer bag containing 90 ml of distilled water and homogenized for 2 min with a stomacher. Water test strips were submerged in homogenized sample in order to determine peroxide value. These procedures were performed three times.

2.8. Statistical analysis

All experiments were replicated three times. Data were analyzed by ANOVA using the Statistical Analysis System (SAS Institute, Cary, NC, USA) and separation of means by the least significant difference (LSD) test. A value of $P \leq 0.05$ was used to indicate significant differences.

3. Results

3.1. Trend of moisture content and water activity of alfalfa seeds according to the time

To ensure the properties of inoculated seeds were as close to the original seed source as possible, moisture content and water activity (a_w) were measured while seeds were being dried in the laminar flow hood. Fig. 1 show changes of moisture content and a_w of alfalfa seeds submerged in peptone water 5 min and dried at 21 °C for up to 24 h. The moisture content and water activity of dried seeds after 20 h was 6.44 ± 0.38% and 0.22 ± 0.02, respectively, and these values were akin to original state of the seeds (6.44 ± 0.21% and 0.23 ± 0.04). This optimum drying time of 20 h was utilized for drying inoculated alfalfa seeds.

3.2. The effect of sequential treatment on inactivation of Salmonella on alfalfa seeds

Inactivation of *Salmonella* Typhimurium on alfalfa seeds subjected to dry heat and hydrogen peroxide solution was dependent on dry heat temperature, exposure time, hydrogen peroxide concentration and soaking time. Preliminary experiments demonstrated that 2% hydrogen peroxide (H_2O_2) treatment for 10 min was optimum because these conditions effectively reduced pathogens while not influencing seed viability. Table 1 shows the correlation of dry heat temperature, time, and hydrogen peroxide for the decontamination of alfalfa seeds. Across all experiments, as dry heat temperature and exposure time increased, the inactivation efficiency also increased.

The initial level of surviving *S.* Typhimurium on inoculated alfalfa seeds was about 10^6–10^7 CFU/g. Treatments of inoculated seeds with 60, 70 or 80 °C dry heat for up to 24 h resulted in 0.71 log, 1.35 log, or 2.76 log CFU/g reductions, respectively. At each dry heat temperature, there was a significant different ($P < 0.05$) between non-dry heated seeds and dry heated seeds. In addition, at 80 °C, the reduction level of 24 h dry heated seeds was significantly different ($P < 0.05$) from that of 12 or 18 h dry heated seeds, but there was no significant difference ($P > 0.05$) at 60 and 70 °C.

Inoculated *S.* Typhimurium populations on alfalfa seeds decreased by 1.41 log CFU/g after treatment with 2% H_2O_2 solution without dry heat. At 60 °C, the inactivation level resulted from H_2O_2 solution treatment after dry heat treatment was not significantly different with that of single treatment H_2O_2. However, at 70 and 80 °C, the sequential treatment inactivated more *S.* Typhimurium at every temperature and time interval. Up to 1.68, 2.83 or 3.60 log CFU/g reduction occurred after 60, 70, or 80 °C dry heat and H_2O_2 sequential treatment, respectively. Sixty and 70 °C, 18 h of dry heat and H_2O_2 treatment reduced *Salmonella* slightly more than other dry heat time treatments at each temperature. However, the difference between dry heat times was negligible.

Soaking in DW following dry heat treatment was performed to determine if there was any washing effect, but there was no significant difference ($P > 0.05$) compared to dry heat treatment alone. This result shows that the additional reduction following H_2O_2 solution treatment was not due to washing.

3.3. The effect of sequential treatments on alfalfa seed viability

Seed viability was evaluated by monitoring the germination percentage, and only seeds that showed an emerging hypocotyl were counted. The germination rate of untreated seeds obtained

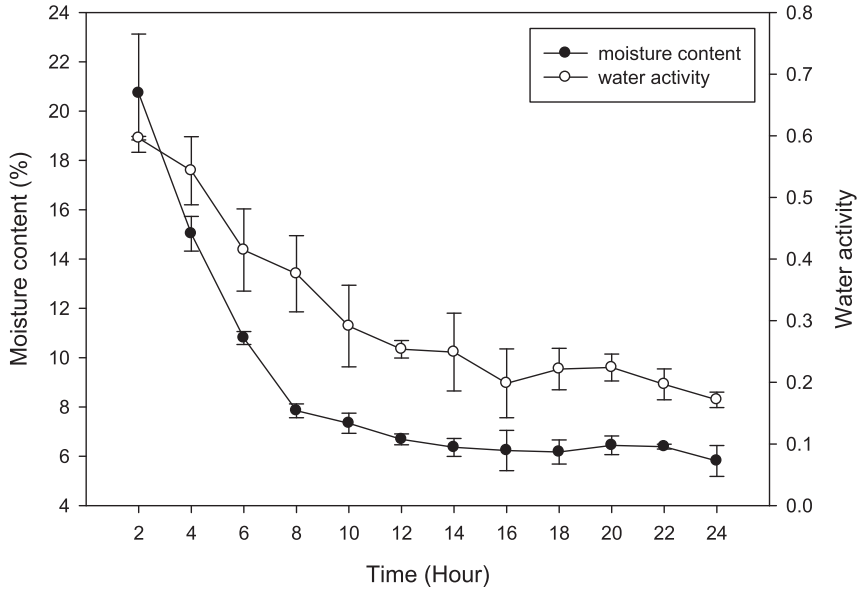

Fig. 1. Moisture content and water activity of alfalfa seeds dried at 21 °C in a laminar flow hood. Alfalfa seeds were submerged in 0.2% peptone water and stirred occasionally for 5 min, then dried for up to 24 h. The moisture content and water activity of control seeds was 6.44 ± 0.21% and 0.23 ± 0.04, respectively.

Table 1
Effect of sequential dry heat and hydrogen peroxide treatments on populations of *Salmonella* Typhimurium on alfalfa seeds.

Temperature (°C)	Treatment	Dry heating time (hours)			
		0[a]	12	18	24
		Population (log CFU/g)			
60	Dry heat only	A 6.61 ± 0.17 Aa	B 6.35 ± 0.37 Aab	B 6.29 ± 0.22 Aab	B 5.90 ± 0.25 Ab
	Dry heat + DW	A 6.53 ± 0.36 Aa	B 6.10 ± 0.27 Aab	B 6.09 ± 0.11 Aab	B 5.93 ± 0.11 Ab
	Dry heat + H_2O_2	A 5.20 ± 0.32 Ba	B 4.95 ± 0.20 Ba	B 4.93 ± 0.13 Ba	B 5.15 ± 0.38 Ba
70	Dry heat only	A 6.61 ± 0.17 Aa	C 5.24 ± 0.24 Ab	C 5.22 ± 0.26 Ab	C 5.26 ± 0.32 Ab
	Dry heat + DW	A 6.53 ± 0.36 Aa	C 5.37 ± 0.24 Ab	C 5.38 ± 0.15 Ab	C 5.02 ± 0.40 Ab
	Dry heat + H_2O_2	A 5.20 ± 032 Ba	C 4.29 ± 0.58 Bb	C 4.16 ± 0.30 Bb	C 3.78 ± 0.56 Bb
80	Dry heat only	A 6.61 ± 0.17 Aa	D 4.83 ± 0.01 Ab	D 4.41 ± 0.20 Ab	D 3.85 ± 0.45 Ac
	Dry heat + DW	A 6.53 ± 0.36 Aa	D 4.78 ± 0.38 ABb	D 4.43 ± 0.21 Abc	D 4.18 ± 0.27 Ac
	Dry heat + H_2O_2	A 5.20 ± 032 Ba	D 3.73 ± 0.83 Bb	D 3.45 ± 0.54 Bb	D 3.01 ± 0.36 Bb

Values represent means and standard deviations of three replications.
Within the table, different uppercase letters to the left of the values indicate significant differences ($P < 0.05$) in populations relative to dry heat temperature.
Within the same column and temperature, different uppercase letters to the right of the values indicate significant differences ($P < 0.05$) in populations relative to dry heating treatment combinations.
Within the same row, different lowercase letters indicate significant differences ($P < 0.05$) in populations relative to dry heating times.
[a] Dry heating time 0 was used as a control to evaluate inactivation levels for dry heat.

from the retailer was 79.5% (Table 2). Dry heat augmented the seed germination percentage by 5.7–13% compared to that of non-dry heat treated seeds. Seeds not treated with dry heat were not significantly different ($P > 0.05$) in germination rate regardless of treatments which followed.

Generally, our results revealed that as dry heat temperature and time increased, the germination percentage increased under all experimental conditions. To put it concretely, treatment with 70 °C dry heat yielded the highest germination rate, all other conditions being equal. Seeds treated with H_2O_2 solution recorded a 0.9–6.5% higher germination than that of untreated seeds. Seeds subjected to 70 °C dry heat for 24 h followed by immersion in H_2O_2 solution achieved a germination of 97.0%, which was the highest percentage of any treatment combination. This figure is 17.5% higher than the control seed groups.

Comparing to the untreated control, a maximum germination increase of 12.2 or 16.2% was observed in the groups treated at 60 or 80 °C, respectively.

3.4. Residual hydrogen peroxide on alfalfa seeds

Residual hydrogen peroxide on alfalfa seeds was measured over time following hydrogen peroxide treatment and after sprouting (Table 3). The detection range of the test strips ranged from 0.05 to 4 ppm; although the test strip might not show the accurate level of residue, it is able to show the trend how the residue level changed over time. The level of hydrogen peroxide residue immediately after treatment was over 4 ppm for every treatment. Over time, however, the levels dwindled, although there were slight differences in the rate of decline. After sprouting, residual hydrogen peroxide levels declined to undetectable levels in every treatment.

4. Discussion

The study was conducted to demonstrate the effectiveness of sequential treatment of dry heat and hydrogen peroxide solution for inactivating *Salmonella* Typhimurium on alfalfa seeds. Although, the US FDA recommends 5 log reductions in pathogen levels, considering the pathogen level of naturally contaminated seeds is less than 1 log CFU/g (Wu et al. 2001; Montville and Schaffner, 2004), a 3.60 log reduction is quite a respectable achievement. In addition, for commercial processing of seeds, a drying step is required after H_2O_2 solution treatment. Kim et al. (2010) reported that further reduction of bacteria was obtained after treatment with sanitizer following by drying at 25 °C. In light of that study, extra reduction was expected when samples were treated with H_2O_2 after heated drying.

Table 2
Germination percentages of alfalfa seeds after sequential treatment of dry heat and hydrogen peroxide solution.

Temperature (°C)	Treatment	Dry heating time (hour)			
		0	12	18	24
		Germination rate (%)			
60	Dry heat only	A 79.5 ± 6.1 Aa	B 86.5 ± 0.9 Ab	B 87.8 ± 0.8 Ab	B 88.3 ± 0.6 Ab
	Dry heat + DW	A 83.0 ± 0.9 Aa	B 88.7 ± 3.2 Ab	B 88.0 ± 0.9 Ab	B 90.8 ± 0.8 Bb
	Dry heat + H_2O_2	A 81.7 ± 1.0 Aa	B 90.3 ± 1.3 Abc	B 89.3 ± 1.0 Ab	B 91.7 ± 0.3 Bc
70	Dry heat only	A 79.5 ± 6.1 Aa	D 88.2 ± 0.8 Ab	D 91.7 ± 1.5 Abc	D 94.3 ± 1.6 Ac
	Dry heat + DW	A 83.0 ± 0.9 Aa	D 90.2 ± 2.3 Ab	D 92.7 ± 1.0 Abc	D 94.0 ± 1.3 Ac
	Dry heat + H_2O_2	A 81.7 ± 1.0 Aa	D 94.7 ± 0.3 Bb	D 95.8 ± 0.8 Bbc	D 97.0 ± 1.0 Bc
80	Dry heat only	A 79.5 ± 6.1 Aa	C 90.2 ± 2.5 Ab	C 91.3 ± 0.8 Ab	C 92.3 ± 0.8 Ab
	Dry heat + DW	A 83.0 ± 0.9 Aa	C 88.7 ± 0.8 ABb	C 92.0 ± 1.7 Ac	C 93.7 ± 0.6 Bc
	Dry heat + H_2O_2	A 81.7 ± 1.0 Aa	C 92.5 ± 0.5 Bb	C 92.2 ± 0.6 Ab	C 95.7 ± 0.6 Cc

Seeds were soaked in distilled water or hydrogen peroxide solution for 10 min after dry heat treatment, then dried at room temperature to restore the condition of an original commercial seeds.
Values represent means and standard deviations of three times replications.
Within the table, different uppercase letters to the left of the values indicate significant differences ($P < 0.05$) in populations relative to dry heating temperature.
Within the same column and temperature, different uppercase letters to the right of the values indicate significant differences ($P < 0.05$) in populations relative to dry heating treatment combinations.
Within the same row, different lowercase letters indicate significant differences ($P < 0.05$) in populations relative to dry heating time.

Table 3
Hydrogen peroxide residue levels on seeds and sprouts after treatments.

Treatment	Residue on seeds (ppm)					Residue on sprouts (ppm)
	Elapsed time after H_2O_2 treatment (hours)					
	0	6	12	18	24	
Control	≤0.05	–	–	–	–	≤0.05
H_2O_2 treatment only	>4.00	1.67 ± 0.58	0.57 ± 0.12	0.22 ± 0.14	≤0.05	≤0.05
60 °C Dry heat + H_2O_2	>4.00	1.67 ± 0.58	0.50 ± 0.20	0.22 ± 0.14	0.13 ± 0.14	0.13 ± 0.14
70 °C Dry heat + H_2O_2	>4.00	1.67 ± 0.58	0.37 ± 0.12	0.22 ± 0.14	0.13 ± 0.14	≤0.05
80 °C Dry heat + H_2O_2	>4.00	2.00 ± 0.00	0.50 ± 0.20	0.22 ± 0.14	≤0.05	≤0.05

Values represent means and standard deviations of three replications.
Dry heating was performed for 24 h, followed byH_2O_2 treatment. H_2O_2residue on seeds was monitored for up to 24 h at 6 h interval.

As mentioned, many studies have been conducted to reduce the level of pathogens on seeds and sprouts. In particular, numerous evaluations have been done to determine the efficacy of chemical treatment including calcium hydroxide, calcium hypochlorite, ethanol, trisodium phosphate, hydrogen peroxide, organic acids, and commercial disinfection agents (Taormina and Beuchat, 1998; Lang et al., 2000). To date, the efficacy of these chemicals has not been shown to be sufficiently adequate to inactivate pathogens attached to alfalfa seeds. Taormina and Beuchat concluded that this is due to cracks and crevices harboring pathogens on alfalfa seed surfaces, where these chemicals couldn't sufficiently contact the pathogens. In addition, according to Charkowski et al. (2001), wrinkled alfalfa seeds harbor more pathogens than smooth seeds, thus pathogens are protected and less impacted by these characteristics of seed morphology.

Dry heat has already been explored as a method for killing pathogens (Bang et al., 2010; Bari et al., 2003). The reason alfalfa seeds were first subjected to dry heat before H_2O_2 solution treatment is because dry heat has not only an inactivating effect but also enables seeds to imbibe more water. Several decades ago studies showed that dry heat makes seeds of the Fabaceae (legume family), including alfalfa, more permeable to water (Rincker, 1954; Lunden and Kinch, 1957). Based on this earlier research, we included dry heat as a drying step before H_2O_2 treatment to maximize the efficacy of hydrogen peroxide by improving penetration underneath the seed coat. We postulated that the synergistic effect might occur in this experiment, but inactivation levels we obtained were a little less than the additional effect. Thus, we concluded that pretreatment with dry heat might not affect to H_2O_2 absorption.

Normally, heat treatments result in diminished seed viability. Yet, alfalfa seed is known to have some degree of heat tolerance. When seeds of this plant undergo heat treatment with moisture, germinability drops in inverse proportion to moisture content. Jaquette et al. dipped alfalfa seeds containing 10^2–10^3 CFU/g of *Salmonella* Stanley in hot water and found they could reduce the population to undetectable levels. However, there was substantial reduction of germination percentage when treated with hot water (>54 °C) for 10 min. Although alfalfa seeds are vulnerable to moist heat, their germination rate can be enhanced by dry heat (Stewart, 1926).

A great number of studies have evaluated the efficacy of chemical sanitizers. These investigations showed that hydrogen peroxide is not the best chemical for inactivating pathogens on seeds. Nonetheless, H_2O_2 solution is approved for sanitizing organic fresh produce, since it doesn't produce any harmful byproduct as it decomposes into water and oxygen, and is designated as GRAS (Lin et al., 2002). Its safety is demonstrated, however, FDA warns consumer against exposing high-strength hydrogen peroxide. It is thus important to monitoring the residual levels of hydrogen peroxide. As our results show, the final product – sprouts –may be safely said to have no residual levels of hydrogen peroxide. This is in harmony with consumer preference for minimally processed foods and organic fresh produce. Moreover, it is reported that H_2O_2 has an effect on increasing seed germination rate in pea seeds, and in cereal plants like wheat (Barba-Espin et al., 2010; Ishibashi et al., 2008). According to the reports, H_2O_2 pretreatment caused an increase the levels of ascorbate oxidizing enzymes which is correlated with increased germination of seedlings. Also, it is said that H_2O_2 treatment alleviates environmental stresses on growth of the radicle and coleoptile. Although the effect of H_2O_2 on germination rate depends on the plant species and the linkage between H_2O_2 and alfalfa seed germination has not been verified, this present study shows that H_2O_2 treatment has some correlation with germination rate. In fact, we observed that radicals were seen through the edge of H_2O_2-treated seeds (data not shown), and those seeds germinated faster than the other samples. There have been a lot of studies in which applied dry heat or hydrogen peroxide; however, those studies have not been reported there was enhanced germination rate (Bari et al., 2003; Feng et al., 2007). The combination of dry heat and hydrogen peroxide; dry heating time and temperature; and hydrogen peroxide concentration might have effected on enhanced germination rate as well as the difference of inherent conditions of seeds and environment.

When both inactivation level and germination rate are considered, treating with dry heat for 24 h at 80 °C is optimum according to results of this study. It is true that treating at 70 °C produced the highest germination rate, but the difference between 70 and 80 °C was only a maximum of 1.3%. This was only an average of 2.6 seeds when 200 seeds were checked for germination. This can cause differentials in economical profit depending on the scale of commercial production. However, since 200 seeds is a fairly small sample size, and the results can vary depending on the seeds sources used, we cannot confirm how representative a difference of 1.3% is between those two treatment temperatures.

Due to morphological characteristics that facilitate trapping of bacterial pathogens beneath the seed coat, and the low water activity (ca. 0.2) of seeds, it was difficult to eliminate *Salmonella* in spite of the hurdle treatment of dry heat and H_2O_2. Unlike other fresh produce and food products which can be consumed immediately following antimicrobial interventions, production of sprouts is complicated by the fact that the final product is produced only after treatment. While dry heat doesn't diminish seed viability (but rather increases the germination rate), long treatment times are required to reduce significant numbers of pathogens. In future studies, other heat treatment methods which can reduce pathogens intensively in a short time while simultaneously maintaining a high germination rate should be studied. Moreover, novel heat treatments or methods that can maximize the efficacy of aqueous chemicals should also be considered.

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