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Short Communication

A comparative study on the effectiveness of chlorine dioxide gas, ozone gas and e-beam irradiation treatments for inactivation of pathogens inoculated onto tomato, cantaloupe and lettuce seeds

V. Trinetta *, N. Vaidya, R. Linton, M. Morgan

Department of Food Science, Purdue University, 745 Agriculture Mall Drive, West Lafayette, IN 47907, USA

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ABSTRACT

The increase in reported food-borne outbreaks linked with consumption of raw fruits and vegetables has motivated new research focusing on prevention of pre-harvest produce contamination. This study evaluates and compares the effectiveness of three non-thermal technologies, chlorine dioxide gas, ozone gas and e-beam irradiation, for inactivation of *Salmonella enterica* and *Escherichia coli* O157:H7 on pre-inoculated tomato, lettuce and cantaloupe seeds, and also their corresponding effect on seeds germination percentage after treatments. Samples were treated with 10 mg/l ClO_2 gas for 3 min at 75% relative humidity, with 4.3 mg/l ozone gas for 5 min and with a dose of 7 kGy electron beam for 1 min. Initial load of pathogenic bacteria on seeds was ~6 log CFU/g. Results demonstrate that all treatments significantly reduce the initial load of pathogenic bacteria on seeds ($p<0.05$). In particular, after ozone gas treatments 4 log CFU/g reduction was always observed, despite the seeds and/or microorganisms treated. ClO_2 and e-beam treatments were noticeably more effective against *Salmonella* on contaminated tomato seeds, where 5.3 and 4.4 log CFU/g reduction were respectively observed. Germination percentage was not affected, except for cantaloupe seeds, where the ratio was significantly lowered after ClO_2 treatments. Overall, the results obtained show the great applicability of these non-thermal inactivation techniques to control and reduce pathogenic bacteria contamination of seeds.

1. Introduction

The number of reported outbreaks associated with the consumption of raw fruits and vegetables has increased in the past few decades. The Center for Disease Control and Prevention (CDC) reported that in more than 50% of outbreaks between 1990–1997 and 1998–2007, the etiological agent was unknown (Buck et al., 2003; CDC, 2010). Outbreaks, where the etiological agent was identified, were primarily of bacterial origin. *Salmonella* spp. and *Escherichia coli* were involved in many food borne illness episodes. These etiological agents can survive for prolonged periods and also at low temperatures, thus presenting an increasing public health concern (Buck et al., 2003). Furthermore, *Salmonella enterica* has recently been associated to tomatoes, sprouted seeds, cantaloupes, apples and oranges, while *E. coli* O157:H7 infections have been associated with lettuce, sprouts, and carrots (CDC, 2010).

Microbial contamination may occur during pre-harvest and/or post-harvest operations. Determining and preventing contamination at all stages of production, harvesting, processing, storage and preparation of fresh and/or fresh-cut produce are important for the economic viability of the food industry and for consumer safety (Beuchat, 2006). Moreover microorganisms can be found in feces, manure, compost, sewage and irrigation water used in fields, where fruits and vegetables are cultivated for consumer use. Studies have demonstrated the transmission of human pathogens from contaminated soil, water, animals and animal wastes to plants (Brandl, 2006; Wachtel et al., 2002; Islam et al, 2005). Solomon et al. (2002) reported, using laser scanning confocal microscopy and epifluorescence microscopy images, that *E. coli* O157:H7 could enter the lettuce plant through the root system and migrate throughout the edible portions.

Few works offered technologies to control and inactivate bacteria attached onto seed surfaces. This approach may represent a valid way to reduce pre-harvest contamination concerns.

Since microorganisms can be introduced at any stage from planting to consumption to delivery of the final product, antimicrobial treatments can provide an effective strategy to control and prevent bacterial food contamination. Washing, rinsing and disinfecting fruits and vegetables are the current techniques used to prolong produce shelf-life. Sanitizers reduce, but do not completely eliminate the number of microorganisms present on food surfaces, since bacteria are able to infiltrate cracks, crevices and intercellular space of produce (Buck et al., 2003). Conversely, the elimination of bacteria from seeds may represent an innovative strategy to reduce the risks of sprout-borne contamination.

* Corresponding author. Tel.: +1 765 494 1212; fax: +1 765 494 7953.
E-mail address: vtrinett@purdue.edu (V. Trinetta).

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The use of non-thermal treatments provides an important intervention to inactivate and control bacteria on seeds without compromising plant development and growth. Some of the more successful antimicrobial non-thermal treatments include chlorine dioxide gas (ClO_2), ozone gas and e-beam irradiation. These techniques are particularly attractive for preharvest decontamination. ClO_2 is a strong oxidizing agent, with a broad antimicrobial spectrum, already applied to decontaminate various fruits and vegetables (Du et al., 2002; Gomez-Lopez et al., 2009). Ozone is also a powerful oxidizing agent, recommended to reduce produce decay and extend storage shelf life (Skog and Chu, 2001). Ionizing radiation represents a technique for pathogen reduction (Black and Jaczynski, 2008; Medina et al., 2009) that involves accelerating electrons almost to the speed of light and subsequently passing them through the matrix in order to inactivate bacteria (Jaczynski and Park, 2003). The electron source is electricity, no radioisotopes are involved, and in addition high dose rates are possible, resulting in a short time exposure.

To date, non-thermal technologies have been used mostly to inactivate food-borne pathogens on seeds intended for direct consumption, such as alfalfa seeds (Sharma et al, 2002) and/or sprouts, like Radish sprouts (Kim et al, 2010) and broccoli sprouts (Waje et al, 2009). Little information is available on the application of these technologies on cultivar seeds (seeds not intended for direct consumption). Therefore, the objectives of this research were to: a) evaluate the effectiveness of ClO_2 gas, ozone gas and e-beam irradiation for inactivation of *S. enterica* Poona and *E. coli* O157:H7 onto tomato, lettuce and cantaloupe seeds, and b) assess the germination percentage of the seeds after each treatment.

2. Materials and methods

2.1. Microorganisms

S. enterica Poona (125) and *E. coli* O157:H7 (EDL 933) strains were selected for this study because of their recent association with fresh produce food borne outbreaks (CDC, 2010). Bacterial strains were obtained from the Purdue University Food Science Department Bacterial Collection (West Lafayette, IN, USA). The strains were grown twice in TSB + 0.6% yeast extract (Tryptic Soy Broth, Difco Laboratories, Spark, MD, USA) at 37 °C to an initial population of approximately 1.0×10^8 CFU/ml.

2.2. Seed inoculation

Cantaloupe (*Cucumis melo* spp. *Melo var. cantaloupensis*), lettuce (*Lactuca sativa*) and tomato (*Solanum lycopersicum* L. *esculentum*) seeds by Ferry-Morse Seed Co (Fulton, KY, USA), with a moisture content of ~3–4%, were selected for the study, purchased from a local supermarket and stored at room temperature until use (25 ± 3 °C). Cantaloupe and tomato seeds, 1000 mg and 300 mg respectively, were inoculated with *S. enterica* Poona (125, Purdue Collection). The seeds, placed in sterile Petri dishes, were soaked with 1 ml of overnight culture, gently stirred for 2 min and then placed in a bio safety cabinet for 2 h to dry and allow for cell attachment (Kim et al, 2010). Similarly, lettuce seeds, 650 mg, were inoculated with *E. coli* O157:H7 (EDL 933) and dried as described above. The seed weights were randomly selected and generally equalled the amount present in one commercial seed packet.

2.3. Treatments

2.3.1. ClO_2 gas treatment

Seeds were treated with 10 mg/l ClO_2 gas for 3 min at 75% relative humidity. Treatment conditions and gas production were based on previous results obtained in our group (Trinetta et al., 2010).

2.3.2. Ozone gas treatment

In another set of experiments, inoculated seeds were treated with ozone gas. Samples were stored in individual re-sealable bags and treatment was performed using a cold plasma, ozone generator (PK-1 system) (Klockow and Keener, 2009). The system (40 W, 60 Hz) generated 12 kV of potential between the electrodes with a concentration of 4.3 mg/l ozone. Gas concentration was measured by using Draeger Short-Term Detector tubes (Draeger Safety AG&Co. KGaA, Luebeck, Germany). Bags containing the samples were filled with oxygen and the electrodes rested on top of each other with the bag in between having an approximate gap distance of 3–3.5 mm. The system was activated for 5 min (ozone generation period) and after treatment, the bags were stored at room temperature for 24 h, prior to microbiological analysis. Treatment times and concentrations were selected according to Klockow and Keener (2009).

2.3.3. E-beam

For irradiation treatments, seed samples were placed in the e-beam Application Development Unit (Advanced Electron Beams, Wilmington, MA) and treated at a fixed energy of 150 keV for 60 s with a dose of 7 kGy. It was important to verify the dose absorbed by the samples, thus the absorbance of cellulose triacetate dosimeters (Far West Technology, Goleta, CA, USA), simultaneously irradiated with the samples, was determined. Since, for majority of foods the radiation dose limit established by FDA is less than 10 kGy (Federal Register, 2005) a radiation dose of 7 kGy was selected.

2.4. Microbiological analysis

At the end of each treatment, samples were collected, diluted in 10 ml of 0.1% peptone water and blended for 5 min (Stomacher 80 Lab-blender, Steward Limited, London, UK). One millimeter of the blended sample was then used to prepare serial decimal dilutions. *Salmonella* population was enumerated using a surface-plate method on XLD agar (Xylose Lysine Deoxycholate, Difco Laboratories, Spark, MD, USA), and plates were incubated at 37 °C for 24 h. To determine *E. coli* population, blended samples were surface-plated on Mac Conkey Sorbitol agar (Difco Laboratories, Spark, MD, USA) and plates were incubated at 37 °C for 24 h. After incubation of plates, colonies on both selective media were counted and standard plate count rules were used to report surviving populations.

2.5. Determination of seed germination percentage

For each seed type, un-inoculated samples (number of seeds = 100) treated with ClO_2, ozone gas and e-beam irradiation at the conditions described in Sections 2.3.1, 2.3.2 and 2.3.3, were placed in a commercial sprout cultivator (Easygreen Factory Co, Sacramento, CA) containing sterile distillated water for 2 weeks at room temperature, 25 ± 3 °C (as indicated by the seller's instruction). The seeds were visually evaluated for germination every day, and sprouted seeds were counted after 2 weeks. The number of seeds that germinated was counted and germination percentage was calculated as described by Kim et al. (2010). The experiment was made in triplicate and un-inoculated and untreated seeds served as a negative control, and used for comparison purpose.

2.6. Statistical analysis

All inactivation experiments, for each seed and treatment type, were performed in triplicate. Non-inoculated samples were considered as negative control, while inoculated samples without treatment were considered as positive controls and used for comparison purpose. Data were analyzed by Multiple Linear Regressions (MLR), employing the RSREG procedure in SAS (SAS 9.1, Inst. Inc., Cary, N.C., USA). Significant differences and comparison among means were

determined using a Tukey multiple comparison test (Minitab 15v, State College, PA). Data were considered to be significantly different when $p<0.05$. Bacterial populations were converted from CFU/ml to log CFU/g. Following conversion, data were pooled and means with standard deviations were calculated and presented in the figure.

3. Results and discussion

The overall aim of this research was to assess the potential applicability of ClO_2 gas, ozone gas and e-beam irradiation as effective non-thermal tools for seed borne pathogen inactivation. Tomato and cantaloupe seeds were each inoculated with *Salmonella Poona* and treated with the three lethal agents in order to evaluate the remaining microbial population. Results are shown in Fig. 1. The initial *Salmonella* population on inoculated and untreated tomato seeds was 5.86 ± 0.09 log CFU/g. Following ClO_2 gas (10 mg/l, 180 s), ozone gas (4.3 mg/l, 320 s) and e-beam irradiation (7 kGy and 150 keV, 60 s), the resulting level of microbial survivors was: 0.54 ± 0.10, 1.80 ± 0.10 and 1.50 ± 0.06 log CFU/g of *Salmonella*. A significant microbial reduction ($p<0.05$) was observed for all of these treatments. A comparative study across treatments was additionally conducted, and the data revealed that ClO_2 gas caused the greatest reduction, followed by ozone gas and then e-beam irradiation. The inactivation data obtained for *Salmonella* population after ClO_2 treatment are in agreement with the 5 log CFU/cm^2 reduction reported for experimentally *Salmonella* inoculated onto Roma tomatoes treated with ClO_2 gas (Trinetta et al., 2010). Different results were reported by Gandhi and Matthews (2003). These authors sprayed a 100 ppm solution of aqueous ClO_2 on alfalfa sprouts contaminated with *Salmonella stanley*, and observed only a 1.5 log reduction after 4 days of growth. Conversely, a combined treatment of 50 ppm ClO_2 and 0.5% fumaric acid significantly reduced *Salmonella typhimurium* by 2.74 log CFU/g on inoculated broccoli sprouts, as compared to the control (Kim et al., 2009). The differences in the results reported are likely due to the different *Salmonella* strains studied, treating seeds versus plant material and the ClO_2 level applied.

Similar to our results found for ozone treatment, Selma et al. (2008) reported a reduction of 4.2 log on inoculated mature non-ripe melons contaminated with *Salmonella*, after treatment with 10,000 ppm ozone gas for 30 min. Our data also showed a 4 log CFU/g *Salmonella* reduction onto tomato seeds after treatments with 4.3 mg/l ozone gas for 5 min. We also found a 4.36 log CFU/g reduction in *Salmonella* population after e-beam treatment of tomato seeds. These results suggest that the effectiveness of ozone gas is not influenced by seeds and or microorganism type.

Several researchers (Taormina et al., 1999; Waje et al., 2009) have already demonstrated the strong inactivation power of ionizing radiation towards human pathogens, showing that irradiation is a promising approach for producing safe and pathogen free seeds and sprouts.

Fig. 1 provides data for the reduction of *Salmonella Poona* on cantaloupe seeds following ClO_2 gas, ozone gas and e-beam treatments. The initial *Salmonella* population was 6.14 ± 0.13 log CFU/g. After treatments, the pathogen counts were reduced to 4.12 ± 0.12, 1.85 ± 0.09 and 3.90 ± 0.09 log CFU/g, with ClO_2, ozone and e-beam, respectively. Unlike tomato seeds, the greatest reduction for cantaloupe seeds was obtained using ozone. The inactivation rate obtained with ozone was significantly different ($p<0.05$) when compared to ClO_2 gas and e-beam treatments. No significant difference ($p>0.05$) was observed between results obtained using ClO_2 gas and e-beam. It is also interesting to note that similar levels of *Salmonella* reduction were reached for both tomato and cantaloupe seeds after treatment with ozone, while both ClO_2 gas and e-beam treatments were noticeably more effective against *Salmonella* inoculated onto tomato seeds, than onto cantaloupe seeds. These results suggest that, unlike ozone, the effectiveness of ClO_2 and e-beam treatments was clearly influenced by the type of seeds.

The initial microbial population of inoculated *E. coli* O157:H7 recovered on untreated lettuce seeds was 5.47 ± 0.14 log CFU/g. Fig. 1 shows the reduction of *E. coli* after treatment. Populations were decreased to 3.53 ± 0.12, 1.94 ± 0.07 and 3.26 ± 0.08 log CFU/g for ClO_2 gas, ozone gas and e-beam respectively. Overall, *E. coli* was more resistant to the lethal treatments compared to *Salmonella* spp. Other researchers (Singh et al., 2003) also indicated the difficulty to completely inactivate *E. coli* O157:H7 from alfalfa seeds. The *E. coli* population observed after ozone treatment on lettuce seeds was 3.53 log CFU/g reduction and this reduction was not significantly different ($p>0.05$) to the result obtained for *Salmonella* contaminated tomatoes and cantaloupe seeds after the same treatment type. This data suggests that the effectiveness of ozone was not influenced by seeds and/or microorganisms strains. In contrast ClO_2 and e-beam were clearly influenced by both of these factors. Waje et al. (2009) studied pathogen inactivation by electron beam and gamma irradiation of commercial seeds sprouts and reported that the efficacy of treatment inactivation depends on the type of pathogen and seeds used. Among the microorganisms analyzed, *E. coli* O157:H7 appeared to be the most resistant.

Overall, the results obtained in the present study reveal that ClO_2 gas, ozone gas and e-beam radiation were all able to significantly reduce the initial load of pathogenic microorganisms on seeds, thereby reducing some pre-harvest contamination concerns. The next step was to evaluate the impact of these treatments on seeds germination and vigor. Table 1 shows the percentage germination of tomato, cantaloupe and lettuce seeds after treatment with ClO_2 gas

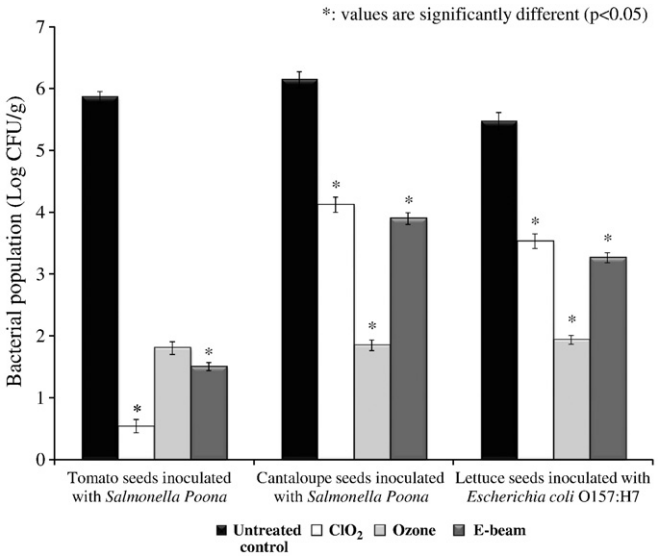

Fig. 1. Inactivation of *Salmonella enterica* Poona (125) on tomato and cantaloupe seeds and *Escherichia coli* O157:H7 (EDL 933) on lettuce seeds after ClO_2 gas, ozone gas and e-beam irradiation treatments. Mean values with standard deviation are presented in the figure. Within each condition, mean values with "*" symbol are significantly different ($p<0.05$).

Table 1
Germination percentages of control and seeds treated with ClO_2 gas (10 mg/l, 180 s), ozone gas, (40 W and 60 Hz, 320 s), e-beam irradiation (70 kGy and 150 keV, 60 s), after 2 weeks of incubation at 25 ± 3 °C.

Germination (%)[#]				
	Untreated control	ClO_2 gas	Ozone gas	E-beam irradiation
Tomato	96 ± 2.5^a	94 ± 1.5^a	94 ± 2.0^a	94 ± 1.0^a
Cantaloupe	92 ± 6.6^a	52 ± 10^b	89 ± 4.9^a	84 ± 8.5^a
Lettuce	95 ± 0.6^a	95 ± 2.9^a	94 ± 3.4^a	91 ± 6.5^a

[#] Mean values (means ± SD) with different letters in the same row are significantly different ($p<0.05$).

(10 mg/l, 180 s), ozone gas (4.3 mg/l for 320 s) and e-beam irradiation (7 kGy and 150 keV, 60 s). Regardless of the seed or treatment type, no significant reduction ($p>0.05$) in germination percent was observed, and sprout vigor (by visual observation) was not compromised. These results indicate the applicability of these treatments as effective seed borne pathogen inactivation control for commercial sprout and seed growers. However, the germination of cantaloupe seeds was significantly affected after treatment with ClO_2 gas, as indicated in Table 1 ($p<0.05$): the germination % recorded was 52 ± 10. Taormina et al. (1999) observed similar findings, reporting that treatment with acidified, aqueous ClO_2 caused the germination percentage of alfalfa seeds to dramatically decrease, while the yield was not affected after treatment with ionizing radiation. Further experiments are required to evaluate the interaction between ClO_2 and cantaloupe seeds, the results obtained in the present study clearly showed the effectiveness of ClO_2 gas, ozone gas and e-beam irradiation for inactivation of *Salmonella* on tomato and cantaloupe seeds and *E. coli* on lettuce seeds.

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