Activity of Electrolyzed Oxidizing Water Against *Penicilium expansum* in Suspension and on Wounded Apples

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ABSTRACT: Spores of *Penicillium expansum*, the primary organism responsible for the occurrence of patulin in apple juice, were exposed to electrolyzed oxidizing (EO) water in an aqueous suspension and on wounded apples. Full-strength and 50% EO water decreased viable spore populations by greater than 4 and 2 log units, respectively. Although EO water did not prevent lesion formation on fruit previously inoculated with *P. expansum*, cross-contamination of wounded apples from decayed fruit or by direct addition of spores to a simulated dump tank was substantially reduced. EO water, therefore, has potential as an alternative to chlorine disinfectants for controlling infection of apples by *P. expansum* during handling and processing operations.

Keywords: *Penicillium expansum*, electrolyzed oxidizing water, apples, patulin

Introduction

Penicillium expansum is the primary cause of postharvest decay of apples (Paster and others 1995). Besides causing economic losses, fruit contaminated with *P. expansum* can accumulate high levels of the mycotoxin patulin (Taniwaki and others 1992). Recently, the U.S. Food and Drug Administration (USFDA) issued a final ruling requiring juice processors to implement Hazard Analysis Critical Control Point plans to control food safety hazards in their operations (Federal Register 2001). The USFDA has issued guidelines for minimizing patulin in apple juice, which include purchasing only sound, tree-picked fruit and culling or trimming after storage to eliminate moldy, damaged, or rotten apples (USFDA 2002b). Of particular concern are small processing operations in which poor-quality apples may be used to make cider (Brackett and Marth 1979). According to current USFDA estimates (USFDA 2002a), if 1 rotten apple containing 10000 parts per billion (ppb) patulin is used along with 200 sound apples to make juice, the resulting patulin level in the juice could exceed USFDA's action level of 50 μg/kg. However, culling may not be completely successful in eliminating patulin from apples because lesions may occur within the interior of the fruit that are not apparent upon visual inspection (Paterson and others 2000). Minimizing inoculum levels and preventing cross-contamination from occurring during handling and storage is, therefore, an important strategy for preventing the occurrence of apple decay and resulting presence of patulin in juice products (Rosenberger 1999).

Chlorine, at levels of 100 to 200 ppm is currently recommended for control of postharvest pathogen spores in dump tanks and other recirculating water systems (Willet and others 1989). However, the use of chlorine has disadvantages, such as the corrosion of metal equipment, reliance on manual monitoring of chlorine concentrations, sensitivity to organic load, effectiveness within a narrow pH range, and the formation of harmful chlorinated byproducts (Roberts and Reymond 1994). Alternative measures for controlling postharvest decay include treating apples with chlorine dioxide (Roberts and Reymond 1994), hydrogen peroxide (Baldry 1983), acetic acid vapor (Sholberg 2000), ozone (Spotts and Cervantes 1992), and calcium salts (Conway and others 1999).

Electrolyzed oxidizing (EO) water is a novel disinfectant that has been shown to effectively reduce pathogenic bacteria in cell suspensions (Venkitanarayanan and others 1999a) on plastic cutting boards (Venkitanarayanan and others 1999b) and on the surface of vegetables (Izumi 1999; Bari and others 2003). Suzuki and others (2002) demonstrated inhibitory activity of EO water on the growth of *Aspergillus parasiticus* and production of aflatoxin. EO water is produced by electrolysis of aqueous sodium chloride to produce an electrolyzed basic aqueous solution containing dilute sodium hydroxide at the cathode and an electrolyzed acidic solution at the anode (Kim and others 2000a).

The activity of EO water against *P. expansum* has not previously been demonstrated. Therefore, the objectives of this study are to determine (1) the activity of EO water against an aqueous suspension of *P. expansum* spores, (2) the efficacy of EO water in preventing decay from occurring on inoculated wounded apples, and (3) the efficacy of EO water in preventing cross-contamination of *P. expansum* introduced into process solutions.

Materials and Methods

Preparation of inoculum

Penicillium expansum (PP497A), an isolate from infected pear fruit, was obtained from the Penn State Fusarium Research Center (Univ. Park, Pa., U.S.A.) and adapted to apple fruit as follows: A 30-mm-wide strip of skin was removed around the equators of 3 fresh apples with a vegetable peeler; 500 μL of a 10^6 colony-forming units (CFU)/mL spore suspension of the isolate was then evenly applied to the exposed flesh, and the inoculated apple incubated at 25 °C for 6 d, during which more than 50% of the peeled strip showed visible decay with spore formation. The procedure was repeated once before inocula from the decayed apples were plated onto potato dextrose agar (PDA) for use in all experiments.

Cultures of the organism were maintained on PDA (Difco,

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Sparks, Md., U.S.A.) slants. Inoculum was produced by growing the organism on PDA slants for approximately 8 d at 25 °C, at the end of which the entire slant surface was covered with spores of the mold. Five milliliters of sterile 0.01% Tween 80 (polyoxyethylene sorbitan monooleate, VWR, Chester, Pa., U.S.A.) were then added to each of the slants, and the tubes were shaken gently to disperse the spores. The suspension was then filtered through 8 layers of cheesecloth to remove mycelial debris. The number of spores was determined using a Bright-Line™ hemacytometer (Hausser Scientific, Horsham, Pa., U.S.A.) and confirmed by PDA plate counts. All reagents and equipment were sterilized by autoclaving at 121 °C for 21 min.

EO water

An ROX™ Water Electrolyzer (Hozishaki America, Inc., Peachtree City, Ga., U.S.A.) was used to generate EO water. A 0.1% sodium chloride solution was electrolyzed in a cell containing inert positively charged and negatively charged platinum electrodes separated by a bipolar membrane composed of vinylidine polyflouride. By subjecting the electrodes to direct current voltage at 19.0 amps, 2 types of water were generated: an electrolyzed basic aqueous solution containing dilute sodium hydroxide (NaOH) produced from the cathode and an electrolyzed acidic solution. Experiments were conducted using the acidic portion.

Determination of free chlorine, pH, and oxidation-reduction potential

Free chlorine content of acidic EO water was measured using a Hach DPD-FEAS digital titrator (Hach Co., Loveland, Colo., U.S.A.) calibrated to measure up to 25 ppm chlorine in a 25-mL solution according to the manufacturer's directions. Briefly, 25-mL samples diluted 10-fold with sterile deionized water were transferred into 50-mL beakers. A DPD-free CL powder pillow was added to each sample and stirred to mix. These were then titrated using 0.00564 *N* ferrous ethylenediammonium sulfate to a colorless endpoint. Free chlorine was calculated from the number obtained following titration, inclusive of the dilution factor. Values for pH and oxidation-reduction potential (ORP) were determined using an Accumet pH meter (model AR25, Fisher Scientific, Pittsburgh, Pa., U.S.A.) with Orion Ion and ORP electrodes (Orion Research, Beverly, Mass., U.S.A.).

Inactivation of spores in suspension

One milliliter of the stock spore suspension containing approximately 10^7 CFU/mL of inoculum was deposited on an individual sterile 0.22-μm cellulose acetate filter fitted onto a 47-mm vacuum filter holder (VWR). Ten milliliters of acidic undiluted EO water or EO water diluted with sterile distilled water to 75%, 50%, or 25% (v/v) of the original concentration was dispensed onto the filter. Sodium hypochlorite (chlorine) solutions containing 100 or 200 ppm chlorine with or without adjustment to pH 7 were included for comparison. Sterile distilled water served as a control. The filter holder was shaken gently to suspend the spores in the treatment solution. Following treatment times of between 15 s and 300 s, the solution was vacuum filtered through the disc, and the remaining spores were washed once with 10 mL of sterile distilled water. The filter was transferred to a 250-mL Erlenmeyer flask containing 50 mL of 0.01% Tween 80 solution, and the spores were dislodged by a 2 min ultrasound treatment in an Aquasonic™ ultrasonicator (VWR) operating at a frequency of 60 Hz. Serial dilutions from the flask were then spread onto pre-poured PDA plates and incubated at 25 °C for 48 h to determine the amount of viable spores. Each experiment consisted of duplicate treatments that were repeated 3 times.

Inoculated apple study

Macintosh apples obtained from a local grocery store were held at 4 °C until ready for use. Before each experiment, 19 apples were washed with mild detergent, rinsed in distilled water, wiped dry with clean paper towels, and allowed to equilibrate to room temperature. An 8-mm circular wound was made to a depth of 3 mm at 5 equidistant locations on each apple using a sterile cork borer and scalpel. Each wounded apple was inoculated by dipping in 500 mL of distilled water containing 10^6 CFU/mL of *P. expansum* spores for 10 s and then air-dried for 1 h. Inoculated apples were then added to a 30-L water bath filled with 20 L of full strength EO water (100% EO), EO water diluted with an equal volume of tap water (50% EO), or 200 ppm chlorine at pH 9.3 or 6.9. Inoculated apples were also held in distilled water as a control.

Conditions in a commercial dump tank were simulated by continuously agitating the apples using a mechanical stirrer and recirculating pump. Following a 5-min treatment, the apples were removed and allowed to dry in air at 25 °C for 1 h. Apples were stored in ventilated plastic bags at 25 °C for 6 d. These experiments were repeated once, and the activity of treatment solutions was expressed as the percent number of wounds showing decay after the storage period.

Cross-contamination study

Nineteen uninoculated, wounded apples were added to the water bath containing EO or chlorine solutions and exposing them to either a single decayed apple or a known concentration of spores. Decayed apples were prepared as described before for the adaptation of *P. expansum* PP497A on fresh apples. For experiments in which spores were added directly to the water bath, the concentration of the stock solution was calculated to achieve approximately the same level of spores released by a decayed apple held for 5 min in the water bath containing distilled water. Following 5-min treatments in EO water or chlorine as previously described, the apples were stored at 25 °C for 6 d, and the percent number of wounds showing decay was determined. All experiments were replicated 2 times.

Statistical analyses

Mean CFU for in vitro treatments and the mean number of infected wounds in apple experiments were compared using 1-way ANOVA procedures. Tukey's HSD test was used to determine significant differences between treatments, and all analyses were performed with Minitab statistical software (Minitab Inc., State College, Pa., U.S.A.).

Results and Discussion

Inactivation of spores in aqueous suspension

The physicochemical properties of EO water or chlorine solutions and reductions in viable *P. expansum* spores after treatment for up to 300 s are shown in Table 1. The pH of undiluted acidic EO water increased from 3.1 to 6.5 after diluting with 3 equal volumes of distilled water. ORP and free chlorine values within the same dilution range decreased from 1133 to 851 mV and 59.6 to 10.1 ppm, respectively. Processors would benefit by using diluted EO water because it would reduce the volume that must be generated while minimizing the potential for equipment corrosion to occur at excessively low pH levels.

The ORP value for unacidified 100 ppm chlorine was less than all EO water solutions. When the pH of each chlorine solution was lowered with HCl, free chlorine remained unchanged while ORP values in each case increased. This can be attributed to the acid induced

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Table 1—Inactivation of *Penicillium expansum* spores by electrolyzed oxidized (EO) water and sodium hypochlorite solutions

	pH	ORP (mV)	Free chlorine (ppm)	Spore inactivation (log_{10} CFU/mL)[a] Time (s) 15	30	60	300
Control	7.5	272	2.2	0.13a	0.28a	0.42a	0.13a
25% EO water	6.5	851	10.1	1.61c	1.70c	2.65d	2.73c
50% EO water	6	895	33.5	2.91d	3.20d	3.42e	3.37d
75% EO water	5	968	50	3.83e	3.84e	4.26f	4.82f
100% EO water	3.1	1133	59.6	4.37f	4.85f	4.82g	4.62f
100 ppm chlorine	8.2	698	100	0.38a	0.74b	1.72b	1.82b
100 ppm chlorine[b]	7.1	900	100	3.39d	3.59e	3.79e	4.41e
200 ppm chlorine	9.8	742	201	0.85b	0.87b	2.00c	4.65f
200 ppm chlorine[b]	6.9	919	200	3.41d	3.88e	4.19f	5.60g

[a]Numbers within each column with different letters indicate significant ($P \leq 0.05$) differences between treatments. ORP = oxidation-reduction potential.
[b]pH was adjusted using 5 *N* HCl.

conversion of hypochlorite ion (OCl^-) to the more reactive hypochlorous acid form (HOCl) (Kim and others 2000b, Eifert and Sangley 2002). ORP values measured at each treatment interval did not significantly ($P \leq 0.05$) change with time (data not shown).

Inactivation of *P. expansum* spores by EO water or chlorine was significantly affected ($P \leq 0.05$) by dilution level or concentration and exposure time (Table 1). Decreases in viable spores were initially rapid with over a 4.6 log reduction observed after 300 s of exposure to undiluted EO water. Unacidified chlorine was initially less effective than EO water, although a greater than 4 log reduction was achieved with a 200-ppm solution after 300 s. Consistent with the positive effect of pH adjustment on ORP values, neutralization of chlorine solutions increased the rate of spore inactivation.

Figure 1 confirms the relationship between ORP of EO water or chlorine solutions and inactivation of *P. expansum* spores. A strong correlation ($R^2 = 0.89$) was observed between ORP and inactivation of spores following exposure to each sanitizer for 60 s irrespective of the treatment used. Kim and others (2000b) demonstrated the relationship between sanitizer effectiveness and ORP and proposed that ORP is a suitable indicator of antibacterial activity. Our findings support a similar conclusion for inactivation of *P. expansum* spores.

A tailing effect observed after 15 s of treatment indicates that prolonged exposure to the sanitizers may not necessarily increase spore inactivation. Further treatment of spores by vacuum filtering the solutions followed by addition of fresh solution, or successive

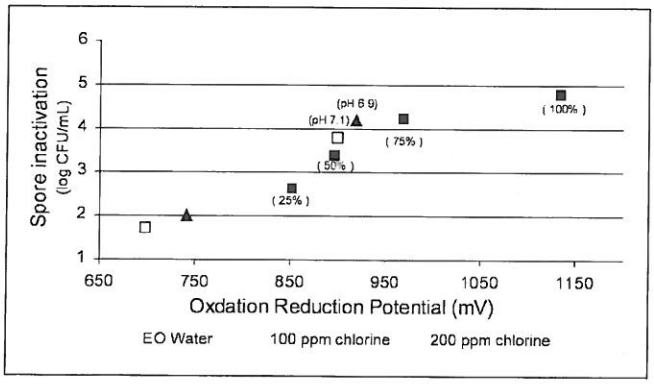


Figure 1—Relationship between oxidation-reduction potential (ORP) of 25%, 50%, 75%, and 100% EO water and chlorine with or without pH adjustment and log_{10} reduction of *Penicillium expansum* spores treated for 60 s.

Table 2—Frequency of decay (%) on inoculated[a] apple wounds treated with electrolyzed oxidizing (EO) water or sodium hypochlorite in a simulated dump tank and stored at 25 °C for 6 d

Treatment[b]	pH	ORP (mV)	Free chlorine (ppm)	Nr of wounds showing decay (%)
Control	7	264	0	100
50% EO water	5.1	927	36.3	100
100% EO water	3	1154	80	100
200 ppm NaOCl	9.3	772	200	100
200 ppm NaOCl[c]	7	897	200	100

[a]Apple wounds were inoculated by dipping in a 10^6 colony-forming units/mL spore suspension for 10 s. ORP = oxidation-reduction potential.
[b]Wounded apples were exposed to treatment solutions for 5 min and stored at 25 °C for 6 d.
[c]pH was adjusted using 5 *N* HCl.

treatment with the alkaline and acid EO fractions did not enhance inactivation (data not shown). This effect is consistent with a study by Kirsten and Nielsen (1995), who found varied resistance to disinfectant treatments among related fungal species. They speculated that fungal resistance to disinfectant agents may be related to the hydrophobic nature of their spore surfaces. The spore suspensions in this experiment were prepared in a solution of Tween 80 to increase their dispersion in the treatment solution. Inouye and others (2001) found that the presence of Tween 80 in liquid media reduced the effectiveness of antimicrobial compounds because of the formation of micelles, which could prevent the interaction of the compounds with spores.

Inactivation of spores on inoculated apple wounds

Table 2 shows the properties of treatment solutions used in water-bath experiments with wounded, inoculated apples. Because 200 ppm chlorine was more effective than 100 ppm in inactivating *P. expansum* spores, this concentration was used for apple treatments. The properties of each solution mirrored those obtained in the previous experiment, in which ORP, for example, was highest for 100% EO water and lowest for the control.

None of the treatments prevented decay lesions from developing on the apples. Mari and others (1999) were similarly unable to prevent decay of nectarines and plums from *Monilinia laxa* using postharvest applications of chlorine dioxide and peracetic acid. In addition, Koffmann and Penrose (1987) failed to prevent *P. expan-*

sum decay on inoculated apples and pears using various fungicides. It is possible that some spores may be embedded within the apple tissue and are thus protected from contact with sanitizers. Interactions between the sanitizer and organic matter on the apple surface may also contribute to this lack of activity.

Prevention of cross-contamination

Addition of a single decayed apple to the water bath resulted in a *P. expansum* spore population of approximately 10^6 CFU/mL. It has previously been shown that *P. expansum* spores can reach 10^3 CFU/mL or higher in apple dump tanks (Spotts and Cervantes 1986). The level used in this experiment would thus simulate severe contamination.

When apples were held in the water bath containing distilled water for 5 min (control), all of the wounds became infected. However, both EO water and 200 ppm chlorine significantly ($P \leq 0.05$) reduced the rate of infection and decay (Table 3). Undiluted EO was more effective than all other treatments, whereas 50% EO water and acidified chlorine had an approximately equal effect. Dilution of EO water with tap water resulted in approximately twice the rate of infection compared with undiluted EO water. When the pH of chlorine solutions was lowered from 10.2 to 7.1, the number of infected wounds decreased by 50%, from 34% to 16.7%.

Despite similar inoculum levels, direct addition of spores into the water bath resulted in higher infection rates compared with addition of a single decayed apple. Because the 10^6 CFU/mL level was determined after a decayed apple was held in the water bath for 5 min, fewer numbers of spores may initially be present as they gradually detach from conidiophores and surrounding mycelial structures. Thus, direct addition of spores to the water bath results in higher numbers of spores that are immediately accessible to the apple wounds.

Table 3—*Penicillium expansum* cross-contamination of uninoculated wounded apples in a simulated dump tank containing electrolyzed oxidized water (EO water) or sodium hypochlorite

				Nr of wounds showing decay (%)[a]	
Treatment[c]	pH	ORP (mV)	Free chlorine (ppm)	Decayed apple[e]	Direct addition of spores[d]
Control	7.4	308	0.8	100a	95.5a
50% EO water	4.7	925	41.9	18.4c	53.3c
100% EO water	2.9	1165	79.1	10.2d	42.3b
200 ppm NaOCl	10.2	755	198	34.0b	51.1c
200 ppm NaOCl[e]	7.1	923	200	16.7c	43.4b

[a]Numbers within each column with different letters indicate significant ($P \leq 0.05$) differences between treatments. ORP = oxidation-reduction potential.
[b]Apples were exposed to treatment solutions for 5 min and stored at 25 °C for 6 d.
[c]Single decayed apple resulted in a *P. expansum* spore population of approximately 10^6 colony-forming units (CFU)/mL in dump tank.
[d]Spore concentration in stock suspension was calculated to achieve 10^6 CFU/mL in the dump tank.
[e]pH was adjusted using 5 *N* HCl.

Conclusions

EO water is a promising alternative to chlorine sanitizers for minimizing postharvest infection of apples in dump tanks and other recirculating water systems. However, the results from this study demonstrate the difficulty in inactivating spores on wounded apples once they have become contaminated. Nevertheless, the use of EO water or other sanitizers in process solutions is warranted because it reduces the potential for cross-contamination to occur in apple handling systems. A possible limitation to the large-scale use of EO water is the cost associated with equipment and electrical consumption. More economical uses that do not require large volumes of water, such as spray applications, may offer savings in cost.

Because treatments to prevent cross-contamination are only partially effective, it is essential to minimize inoculum levels and thus reduce the incidence of patulin in juice by harvesting only tree-picked apples, handling them carefully to avoid wounding, and following a regular cleaning and sanitizing program in handling and storage facilities.

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