Survival of *Penicillium expansum* and Patulin Production on Stored Apples after Wash Treatments

L. CHEN, B. H. INGHAM, AND S.C. INGHAM

ABSTRACT: *Penicillium expansum* is a widespread fungus found on apples that causes fruit decay and may lead to production of a toxic secondary metabolite, patulin. This study was undertaken to evaluate the effectiveness of several chemical sanitizers against *P. expansum* NRRL 2304 and to establish sanitizing wash treatments that would inhibit *P. expansum* growth and subsequent patulin production on Empire apples destined for cider. Wash treatments included 200 ppm NaOCl, 1% StorOx®, 0.5% potassium sorbate, 300 ppm SO_2, and 0% to 5% acetic acid. Spores of *P. expansum* or inoculated apple slices were dipped in sanitizing wash solution for 5 min, and mold growth and patulin production was monitored on subsequent storage. It was found that 0.5% potassium sorbate and 300 ppm SO_2 did not affect mold survival or patulin production; 1% StorOx® was effective against mold spores in solution (4 log Most Probable Number destruction of spores), but there was no significant reduction in spore count when the same solution was used to sanitize mold-inoculated apple discs. Washing with 200 ppm NaOCl delayed growth of *P. expansum* on inoculated apple discs but failed to completely inhibit patulin production. Acetic acid solution (2% to 5%) was the most efficient chemical against *P. expansum*. A wash treatment with ≥2% acetic acid for more than 1 min is recommended to completely inhibit growth of *P. expansum* and subsequent patulin production on apples destined for cider.

Keywords: patulin, *Penicillium expansum*, washing treatment

Introduction

Patulin, 4-hydroxy-4H-furo-(3, 2c)-pyran-2-(6H)-one, is a toxic secondary metabolite produced by certain species of *Penicillium*, *Aspergillus*, and *Byssochlamys* (Harrison 1989). Animal studies have revealed that patulin exhibits carcinogenic, teratogenic, and mutagenic effects in rodents (Mayer and Legator 1969; Ciegler and others 1976; Hopkins 1993). Among patulin-producing fungi, *Penicillium expansum* is the strain most commonly found in apples, leading to substantial fruit loss and establishing a potential health hazard due to soft "blue mold rot" decay and patulin production (Doores 1983).

Apples and apple products are the major human dietary sources of patulin (Stott and Bullerman 1975). Surveys have revealed frequent occurrences of patulin in apple juice (Lindroth and Niskanen 1978; Brackett and Marth 1979b; Wheeler and others 1987; Burda 1992; Yurdun and others 2001), leading to increased safety concerns regarding the implications of human exposure to high levels of patulin. Consequently, an action level of 50 μg/L for patulin in apple juice has been established in the United States (USFDA 2002a). Furthermore, the United States Food and Drug Administration (USFDA) has issued guidelines for minimizing patulin in apple juice, including the culling or trimming of apples after storage to eliminate moldy, damaged, or rotten apples (USFDA 2002b).

In addition to methods such as culling, Good Agricultural Practice (GAP) guidelines currently recommend a wash of at least 150 ppm active chlorine for crops postharvest to reduce microbial contamination and extend shelf life (USFDA 1998). The disadvantages of chlorine, such as extensive corrosion of metal equipment, sensitivity to organic load, effectiveness over a relatively narrow pH range, and the formation of chlorine by-products, have led researchers to consider other treatments with which to remove postharvest pathogens from fruit. Treatments that have been evaluated for effectiveness against *P. expansum* on apples include chlorine dioxide (Roberts and Reymond 1994), cinnamon oil or potassium sorbate (Ryu and Holt 1993), acetic acid vapor (Sholberg and others 2000), a combination of heat, calcium infiltration, and competitive exclusion (Conway and others 1999), and electrolyzed oxidizing water (Okull and LaBorde 2004). Modified atmosphere packaging of individual apples has also been investigated (Moodley and others 2002). These treatments have proven to be effective to varying degrees.

Sydenham and others (1997) showed that if apples are stored in the open ("deck storage") for extended periods (33 d) before being processed into juice, significant amounts of patulin can remain in the juice even after washing and culling of fruit (55 to 405 ng/g). And it is possible that a wash treatment, rather than having the desired effect, may serve to increase contamination on apples (Lang and others 1999). Accordingly, methods for reducing patulin levels in apple juice or cider have been studied. Physical methods such as centrifugation and filtration (Bissessur and others 2001) and absorption with activated carbon in a "static" or "flow through" system have been evaluated (Huebner and others 2000; Gokmen and others 2001). Adoption of these types of methods by processors is hindered due to their limited effectiveness and also due to the adverse quality changes that they produce in the juice. Thermal and chemical treatments of juice to destroy patulin have also been investigated. High-temperature short-time processing will re-

duce, but not destroy, the toxin (Wheeler and others 1987; Kadakal and others 2002). When chemical treatments are considered, research by Brackett and Marth (1979a) showed that when ascorbic acid was added at up to 5% (w/v) to patulin-spiked apple juice, the patulin disappeared from the assay system. However, the overall effectiveness of physical, thermal, and chemical treatments for the removal or destruction of patulin from apple juice cannot be determined until research establishes that no new toxic products result from such treatment. Thus, preventing patulin from contaminating apple juice is of primary importance.

No studies to date have evaluated the effectiveness of wash treatments against *P. expansum* and the ability of these treatments to prevent patulin from contaminating juice made from stored apples. Therefore, the objectives of this study were to screen commonly used sanitizers for effectiveness against *P. expansum* on apples and to establish effective sanitizing wash treatments that both inhibit *P. expansum* and prevent the formation of patulin on stored apples destined for cider.

Materials and Methods

Apples

Empire apples were obtained from an orchard near Madison, Wis., U.S.A., in October 2003, and stored in a walk-in cooler at 4 °C before use.

Culture preparation

Penicillium expansum NRRL 2304 was obtained from the United States Dept. of Agriculture Agricultural Research Service (Natl. Center for Agricultural Utilization Research, Peoria, Ill., U.S.A.). Strain NRRL 2304 is a known patulin producer. The culture was maintained on potato dextrose agar (PDA; Difco, Sparks, Md., U.S.A.) at 25 °C. Spore suspensions were prepared by flooding PDA plates (1 wk old with visibly abundant spore formation) with 10 mL of sterile water and swirling gently to release the spores. The number of spores in suspension was enumerated by the Iso-Grid® system (Neogen, Lansing, Mich., U.S.A.). Briefly, liquid samples were properly diluted with Butterfield phosphate buffer (Nelson Jameson Inc., Marshfield, Wis., U.S.A.) and passed through the Iso-Grid® membrane filtration unit under vacuum. Most of the mycelial mass and other debris were trapped on the prefilter, whereas mold spores passed through the prefilter and were trapped on the membrane below. Sterile water was passed through the filtration unit, if necessary, to wash away chemical sanitizers trapped on the membrane. The membrane filter, with spores entrapped, was aseptically placed, grid side up, onto the surface of a YM-11 agar plate (Neogen). After 50 h of incubation in an inverted position at 25 °C, the number of blue colonies on the membrane filter was enumerated as the "score." Subsequently, the score was converted to a Most Probable Number (MPN) using an MPN Conversion Table (Appendix A, Neogen Corp. 1999).

Determination of pH

The pH of wash solutions and apples was measured with an Accumet Basic AB15 pH meter (Fisher Scientific, Chicago, Ill., U.S.A.). An Accumet surface electrode (Fisher Scientific) was used to measure the surface pH of apple discs, before or after sanitizing wash treatment, and was reported as an average of 3 measurements per disc.

Apple inoculation

Preliminary experiments were used to establish the method of apple inoculation as follows:

Trial 1. Sound apples were dipped for 5 min into various dilutions (10^2 to 10^6 MPN/mL) of spore suspension, allowed to air-dry, and then stored at room temperature, approximately 20 °C. Mold growth and the development of rotten tissue was monitored over 2 wk. The intact skin apparently protected the apple tissue, which failed to become infected.

Trial 2. Stab wounds (0.5 cm deep and 0.5 cm in dia) were introduced into sound apple tissue by puncturing the skin with a sterile nail at 4 spots evenly distributed on each apple. Punctured apples were then dipped into mold spore suspension for 5 min, allowed to air dry, and stored at room temperature for 2 wk. Most of the apple wounds became infected, as measured by the diameter of rotten tissue, but the level of infection varied dramatically. This variability may have been due to the inconsistency with which the spores contacted and subsequently colonized the wounded tissue. Because this method of inoculation did not yield consistent results, other methods were investigated.

Trial 3. Rather than dipping stab-wounded apples into a spore suspension, a fixed amount of spores (10^2 MPN) were pippetted into stab wounds. Although this solved the problem of inconsistent inoculation, contact between the spores and the wash solution was still inconsistent. Because of the overall difficulty in designing a whole-apple system of spore inoculation, we subsequently adopted a method of inoculating and treating apple slices to evaluate the effectiveness of sanitizers in inhibiting *P. expansum* and preventing subsequent formation of patulin.

The method of apple inoculation that we designed was as follows: Empire apples were equilibrated to room temperature and surface-sterilized with 70% ethanol. The apples were cut horizontally into discs of 1 cm thickness (about 20 g) using a sterile knife, the skin remaining intact. For inoculation, a 0.1-mL spore suspension containing 10^4 MPN/mL of *P. expansum* was spread onto 1 side of the apple disc. The disc was allowed to air-dry for 1 h before treatment to allow spore attachment and to remove visible moisture. Growth of *P. expansum* on apple discs at 25 °C was monitored for 20 d.

Effect of sanitizing wash treatment

The following 6 wash solutions were screened for their effectiveness against *P. expansum*: sterile distilled water; 200 ppm NaOCl (Fisher Scientific); 1% (v/v) StorOx® (27% active hydrogen peroxide; BioSafe Systems, Glastonbury, Conn., U.S.A.); 0.5% (w/v) potassium sorbate (Fisher Scientific); 300 ppm SO_2 (prepared by dissolving sodium metabisulfite [Fisher Scientific] in sterile distilled water); and 5% acetic acid (v/v) (Fisher Scientific). To further evaluate the effectiveness of acetic acid wash, other concentrations (0.1%, 0.5%, 1%, 2%, 3%, and 5%) were also evaluated.

Initially, the effect of these sanitizers on spores of *P. expansum* in vitro was determined. Spore suspension (0.1 mL, 10^7 MPN/mL) was added to 20 mL of sanitizing wash solution and allowed to stand for 5 min. A 5-min contact time was selected as sufficient to produce an effect, and considered realistic in an apple-processing environment. After treatment, viable spores in the wash solution were enumerated with the Iso-Grid® system as previously described. The Iso-Grid membrane (with spores attached) was washed with 50 mL sterile distilled water to eliminate any chemical residue that may have persisted on the filter during incubation.

On inoculated apples: Two inoculated apple discs (prepared as described previously) were treated by dipping into wash solution (1 L) and held fully immersed for 5 min with gentle agitation. Inoculated apples that were not washed served as the positive control. Treated apple discs were allowed to surface-dry for up to 1 h after wash treatment until visible moisture was gone, and placed in sterile petri dishes (1 slice per dish, inoculated side up) and incubated

at 25 °C for 7 d. The 7-d incubation period was adequate for mold growth, as measured by spore production and patulin production (discussed subsequently).

After incubation, 100 mL sterile water was added to each apple disc in a sterile Whirl-pak filter bag (15 × 23 cm, Fisher Scientific) and homogenized for 2 min in a Model 400 Stomacher (Seward, Norfolk, U.K.). Viable mold spores in the homogenate were enumerated using the Iso-Grid® system. To determine the effect of each wash treatment on production of patulin, inoculated apple discs, treated as described previously, were homogenized and assayed for patulin production at the end of the incubation period. All experiments were conducted in duplicate.

Extraction of patulin

Patulin was extracted from apple tissue by the method of Brause and others (1996) with modifications. Briefly, apple discs were extracted with methyl acetate (Fisher Scientific) 3 times, 20 mL each time, by grinding in a mortar with pestle. The resulting solvent extracts were combined, and 20 mL was removed for further processing. Two milliliters of a 1.5% sodium carbonate solution (Fisher Scientific) was added to the extract to remove co-extracts that might interfere with the analysis. The methyl acetate phase was then collected and dried with 1 g anhydrous sodium sulfate (Fisher Scientific). The solvent was evaporated at 40 °C under a stream of nitrogen, and the residue was subsequently dissolved in 1 mL of pH 4 acetic acid solution (Fisher Scientific). The acetic acid extracts were passed through a 0.22-μm Millipore syringe filter (25-mm dia, Fisher Scientific) before high-performance liquid chromatography (HPLC) analysis.

Patulin assay

Patulin content was determined by the method of Brause and others (1996). A Hewlett Packard 1100 HPLC (Agilent, New Castle, Delaware, U.S.A.) equipped with a PDA detector was used to identify and quantify patulin in apples. Ten microliters of sample was injected. Separation was conducted at room temperature on a Discovery C18 column (250 × 46 mm, 5-μm pore size; Supelco, Bellefonte, Pa., U.S.A.) protected by a Supelguard Discovery C18 guide column (20 × 4 mm; Supelco). The mobile phase was water/acetonitrile (90:10) (HPLC grade, Fisher Scientific) at a flow rate of 1 mL/min. Patulin concentration was calculated in reference to the peak area (wavelength set at 276 nm) of patulin working standard solution (patulin =98%; Sigma-Aldrich, Milwaukee, Wis., U.S.A.). The working standard solution (100 μg/mL) was prepared in acetic acid solution (pH 4) and serially diluted to 100, 80, 60, 40, 20, and 0 μg/mL and analyzed by HPLC, yielding a standard curve that was linear between 20 and 100 μg/mL with an R^2 value of 0.99 (data not shown).

Statistical analyses

Statistical analysis of the data was conducted using the Statistical Analysis System (SAS Inst., Cary, N.C., U.S.A.). Data means were compared with the least significant difference (LSD) test. The significance level was set to 0.05.

Results and Discussion

Growth of *Penicillium expansum* and production of patulin on Empire apples

The waxy skin of a sound apple cannot readily be penetrated in the environment and is a natural protection against mold infection. As a result, infection of apples with pathogens such as *P. expansum* normally occurs in "drops" or in apples that are damaged by hail, insects, birds, or handling (Sydenham and others 1995). We devised a system using freshly cut Empire apple discs to evaluate the growth of *P. expansum* by measuring spore production. Inoculated apple discs were incubated at 25 °C for up to 20 d and spore production was monitored (Figure 1).

Maximum mold spore production on Empire apple slices was reached in approximately 6 d at 25 °C. McCallum and others (2002) monitored the increase in mycelial dry weight to evaluate growth of various strains of *P. expansum* in apple ciders made from different apple varieties. They noted that mold growth was affected by apple pH, sugar content, and temperature. Empire apples used in this study had an average surface pH of 3.5 and sugar content of 12.8 °Brix in pressed apple juice (data not shown), consistent with parameters that would support mold growth and patulin production (McCallum and others 2002). In our study, mold growth and spore production corresponded to visible deterioration in apple tissue compared with the control. Marked tissue browning and softening occurred, accompanied by some moisture loss and shrinkage of tissue.

Patulin production was monitored to evaluate the correspondence between mold spore production and toxin production (Figure 2). Patulin was detected in mold-inoculated apple slices after 2 d, and increased to 950 μg/g apple disc within 20 d. Rychlik and
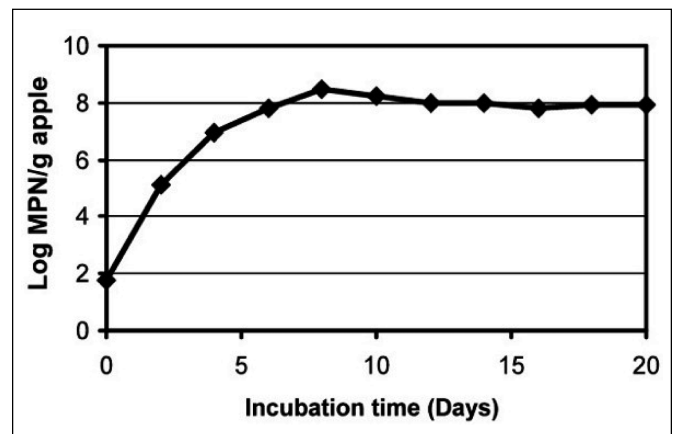
Figure 1—*Penicillium expansum* **NRRL 2304 spore production on the freshly cut surface of Empire apple discs (25 °C)**
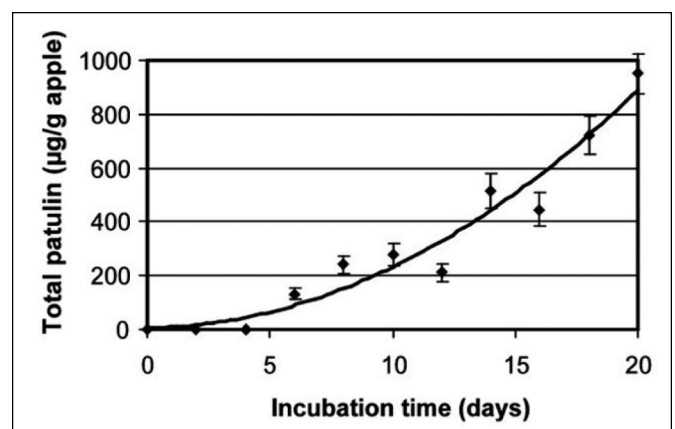

Figure 2—Patulin production by *Penicillium expansum* NRRL 2304 on the freshly cut surface of Empire apple discs (25 °C)

Schieberle (2001) noted that patulin production at 20 °C in Golden Delicious apples began 2 d after inoculation with a strain of *P. expansum*, whereas Sydenham and others (1997) found that patulin levels in juice freshly pressed from deck-stored apples increased dramatically, from 90 ng/g on day 7 to 2445 ng/g after 33 d of storage. McCallum and others (2002) showed that patulin production in mold-inoculated juice made from Empire apples, after an initial lag, increased over a 14-d incubation period and was preceded by a drop in juice pH to a value more favorable for toxin production. Other researchers have noted that the maximum pH for patulin production by *P. expansum* ranged from 3.2 to 3.8 in apple juice (Damoglou and Campbell 1985).

Therefore, it appears that the patulin content of mold-contaminated apple tissue is related to both the population of mold spores and to the length of time that *P. expansum* has been growing. These results support the premise that patulin is a nonessential secondary metabolite whose production may provide *P. expansum* with a competitive advantage over other species. Its production may also be linked to nutritional stress (McCallum and others 2002).

Screening of wash solutions

Survival of *P. expansum* in various sanitizing wash solutions. Various wash solutions were evaluated as sanitizers against *P. expansum* (Table 1). Survival of spores after 5 min contact with sanitizing solution is shown in Figure 3.

Potassium sorbate (0.5%) did not inactivate spores of *P. expansum,* whereas 200 ppm NaOCl and 300 ppm SO_2 decreased spore populations by approximately 1 log MPN/mL. Statistically, the effect of either 0.5% potassium sorbate, 200 ppm NaOCl, or 300 ppm SO_2 was not significantly different from the negative control (sterile distilled water).

Several studies have described the effectiveness of sorbic acid and its potassium salts as preservatives against fungi in culture media. Sorbic acid, when added at concentrations up to 0.025% as part of the culture medium, was reported to stimulate the growth of *P. expansum* and secretion of patulin, with higher concentrations leading to a decrease in toxin production relative to the control (Podgorska 1992). Lennox and McElroy (1984) also showed that potassium sorbate added to nutrient broth at 0.3% reduced growth
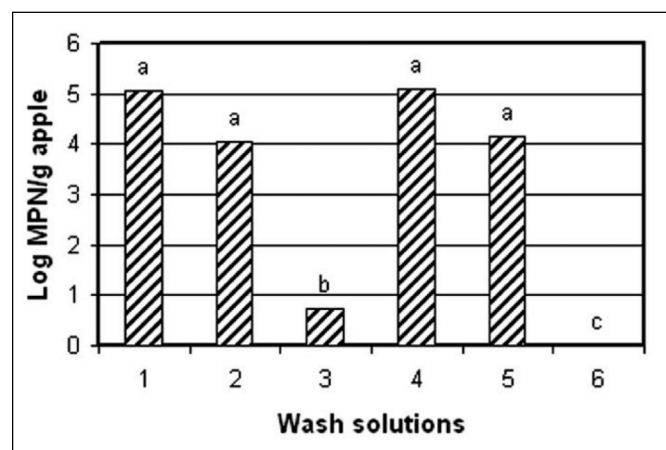
Table 1—Wash solutions (and pH) evaluated as sanitizers against *Penicillium expansum* NRRL 2304

Nr	Wash solution	pH
1	Sterile distilled water (control)	5.5
2	200 ppm NaOCl	9.7
3	1% StorOx®	3.3
4	0.5% Potassium sorbate	7.5
5	300 ppm SO_2	4.3
6	5% Acetic acid	2.4

of *P. expansum* by 57% and patulin synthesis by 67%. Increasing the concentration of potassium sorbate to 1.5% in the growth medium inhibited patulin production by 98% (Lennox and McElroy 1984). In our study, no inhibitory effect was observed for 0.5% potassium sorbate, perhaps due to the short contact time: 5 min for wash solution versus up to 12 d in culture media.

Chlorine and a number of hypochlorites are widely used as sanitizers in the food-processing industry. Good Agricultural Practice (GAP) guidelines currently recommend a postharvest wash treatment of fresh produce in 50 to 200 ppm total chlorine solution, at a pH of 6.0 to 7.5, with a contact time of 1 to 2 min (USFDA 1998). In our study, spores of *P. expansum* were not significantly affected by treatment with 200 ppm NaOCl. Similarly, work by Venturini and others (2002) reported that sodium hypochlorite solution, in concentrations as high as 1000 ppm, did not exhibit an inhibitory effect on *P. expansum* in an agar diffusion assay, perhaps due to the inability of the chlorine to diffuse through the agar medium. Subsequently, however, when sodium hypochlorite was added to a broth dilution assay system, Venturini and others (2002) found that 250 ppm chlorine effectively inhibited growth of the mold by 80% over 9 d, and addition of 1000 ppm chlorine to broth inhibited growth of the mold by nearly 100%. More recently, Okull and LaBorde (2004) noted that 200 ppm chlorine and acid-adjusted 200 ppm chlorine (pH adjusted to 6.9) were effective at inactivating spores of *P. expansum* over a 5-min contact time in aqueous suspension. The effect was more pronounced and more rapid with pH-adjusted (neutralized) chlorine. In our study, we did not adjust the pH of the chlorine solution, resulting in a treatment solution of pH 9.7. Eifert and Sanglay (2002) pointed out that at this pH, most of the chlorine would be in the form of hypochlorite ion, a relatively ineffective antimicrobial compound. Thus lack of effectiveness in this study was probably due to the high pH of the treatment solution, further emphasizing the need to treat produce with pH-adjusted chlorine as the USFDA recommends (USFDA 1998). Small apple processors in particular, who may not have ready access to chemical supply companies, should be advised of the importance of pH in the antimicrobial effectiveness of sodium hypochlorite.

SO_2 at 300 ppm has been shown to effectively inhibit the growth of *P. expansum*, and subsequent patulin secretion, when present in a broth/agar medium for an extended period (12 d) (Podgorska 1992). However, our study indicated that short-time contact with 300 ppm SO_2 (5 min) did not significantly damage spores of *P. expansum*.

StorOx®, containing 27% hydrogen peroxide as an active ingredient, is a commercial postharvest treatment for fruits and vegetables. The concentration used in our study (1% StorOx®) was specifically recommended by the manufacturer against spores of *P. expansum*, and treatment resulted in a 4 log MPN/mL reduction in mold spores. In a broth assay system, a concentration of at least 0.025% hydrogen peroxide has been shown to completely inhibit the growth of P. *expansum* (Venturini and others 2002).

Figure 3—Survival of *Penicillium expansum* NRRL 2304 spores (10^6 Most Probable Number [MPN]/mL) after a 5-min contact with various sanitizing wash solutions: 1 = sterile water (control); 2 = 200 ppm NaOCl; 3 = 1% StorOx®; 4 = 0.5% potassium sorbate; 5 = 300 ppm SO_2; and 6 = 5% acetic acid. Bars labeled with different letters indicate significant differences ($P < 0.05$) between treatments.

Acetic acid (5%) was the most effective treatment against spores of *P. expansum* in our study, with no spores recovered after a 5-min treatment. Venturini and others (2002) noted that acetic acid was lethal for spores of *P. expansum* at concentrations of at least 0.25%, and the effective use of this chemical for treatment of fruit post-harvest has been demonstrated (Sholberg and others 2000).

Survival of *P. expansum* and patulin production associated with inoculated Empire apple discs after wash treatment. Efficacy of the chemical wash treatments differed when Empire apple discs were inoculated with spores of *P. expansum*, allowed to air-dry, and treated (Figure 4). Acetic acid (5%) was the only treatment that yielded a significant reduction in the population of *P. expansum* on apple discs. Treatment with 200 ppm NaOCl did delay the growth of mold on apple discs in the 1st 2 d, as judged by the development of decay and brown lesions on the apple tissue (data not shown), but the mold population was unaffected at the end of the 7-d incubation period, relative to the untreated apple discs. Okull and LaBorde (2004) also reported that chlorine at various concentrations (100 to 200 ppm) did not prevent decay lesions from developing on *P. expansum*–infected apples. Chlorine (200 ppm) and 200 ppm acidified chlorine (pH adjusted to 7) were ineffective against spores of *P. expansum* that had been inoculated onto wounded Macintosh apples. Okull and LaBorde theorized that the ineffectiveness of chlorine may have been due to spores embedded within the apple tissue and thus were protected from contact with sanitizers, or due to interactions between the chlorine and organic matter on the apple surface.

Because the USFDA has set action limits for patulin in apple juice, it is important that any effective sanitizer not only inhibits the growth of *P. expansum* but also prevents the production of patulin in apple tissue during storage. Patulin production by *P. expansum* on apple discs 7 d after wash treatment is shown in Figure 5. Patulin level was not significantly different between the no-wash treatment and wash treatments with sterile water, 1% StorOx®, 0.5% potassium sorbate, and 300 ppm SO_2. The 200 ppm NaOCl wash treatment led to significantly lower patulin content compared with no-wash treatment. Considering the lag time of patulin production (Figure 2), the delay in mold growth caused by treatment with 200 ppm NaOCl (data not shown) could account for the lower level of patulin detected. However, because our results indicate that the effect of 200 ppm NaOCl on mold growth was no longer apparent after 7 d at 25 °C (Figure 4), patulin content could be expected to increase over time, rendering treatment with 200 ppm NaOCl ineffective. Not surprisingly, patulin was not detected on apple discs after treatment with 5% acetic acid because no spores of *P. expansum* survived the acid wash.

Efficacy of acetic acid as a sanitizer for apple processing

Because our results indicated that acetic acid was an effective sanitizer for apple tissue, which could inhibit production of patulin in apple tissue, we chose to focus further studies on the efficacy of this chemical as a sanitizer of potential importance for the apple-processing industry.

Concentration of acetic acid wash solution. The effectiveness of acetic acid wash solutions of various concentrations (0%, 0.5%, 1%, 2%, 3%, or 5%) against *P. expansum* spores was evaluated. Spores of *P. expansum* were placed in solutions of acetic acid ranging from 0.5% to 5%; after 5 min, the spores were removed and survival was evaluated (Figure 6). Decreasing the concentration of acetic acid dramatically reduced the effectiveness of the acid at inhibiting survival of *P. expansum*. A 5-min treatment with an acetic acid solution of ≤2% was ineffective at reducing the mold population.

When acetic acid solutions of the same concentration were used as sanitizing wash solutions for *P. expansum*–inoculated apple discs, no spores were detected on apple discs after 7 d of incubation when acetic acid wash concentrations of 2%, 3%, or 5% were used (Table 2). Simultaneously, no brown lesions were observed on inoculated apple tissue washed with acetic acid at concentrations of at least 2% and, consequently, no patulin was detected. There was a noted increase in the effectiveness of 2% or 3% acetic acid as a sanitizer
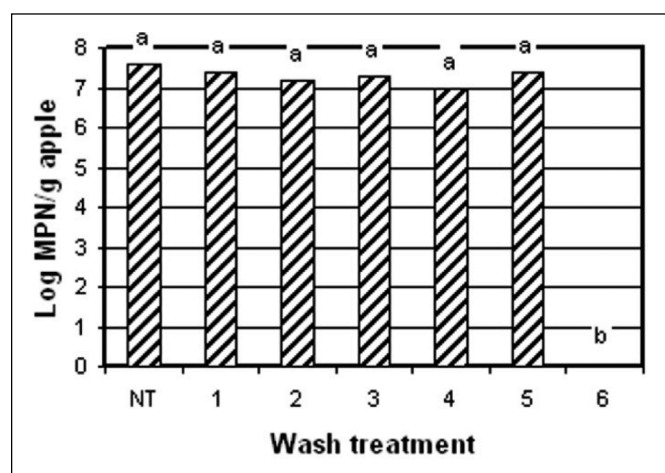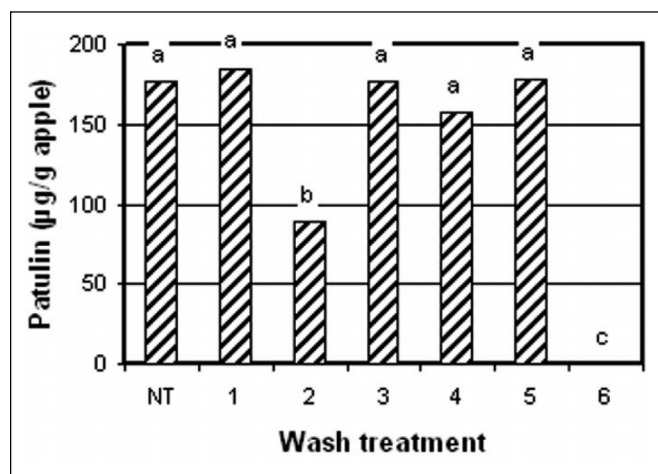
Figure 4—Survival and growth of *Penicillium expansum* NRRL 2304 spores (10^3 Most Probable Number [MPN]/mL) on Empire apple discs after 5 min of treatment in various sanitizer solutions, measured after 7 d of incubation at 25 °C. NT = no wash treatment. 1 = sterile water; 2 = 200 ppm NaOCl; 3 = 1% StorOx®; 4 = 0.5% potassium sorbate; 5 = 300 ppm SO_2; and 6 = 5% acetic acid. Bars labeled with different letters indicate significant differences between treatments.

Figure 5—Effect of various sanitizer treatments on production of patulin by *Penicillium expansum* NRRL 2304 (inoculum of 10^3 spores) in vivo. *P. expansum* was inoculated onto Empire apple discs; the discs were allowed to air-dry and then treated for 5 min in various sanitizer solutions. Patulin production was monitored after 7 d at 25 °C. Sanitizer wash solutions: NT = no wash treatment. 1 = sterile water; 2 = 200 ppm NaOCl; 3 = 1% StorOx®; 4 = 0.5% potassium sobate; 5 = 300 ppm SO_2; and 6 = 5% acetic acid. Bars labeled with different letters indicate significant differences between treatments.

against *P. expansum*–inoculated apple tissue when compared with a liquid system (Figure 6). This increased effectiveness may be due to acid residue remaining on the inoculated apple discs after the wash treatment. When apple discs were washed with less than 2% acetic acid, mold growth was not completely eliminated and patulin production was detected at the end of 7 d. These results indicate that an apple wash treatment with ≥2% acetic acid could be an effective sanitizer against *P. expansum*.

Treatment times of acetic acid wash solution. To establish the full set of parameters for effective application of an acetic acid wash solution in a Hazard Analysis Critical Control Point (HACCP) program, it is necessary to specify both treatment concentration and time. The effect of treatment time on *P. expansum* spores in 5% acetic acid is shown in Figure 7. Treatment time played an important role in the inactivation of mold spores in 5% acetic acid, with effectiveness ranging from no apparent inhibition of spores for treatment under 1 min, to a reduction of at least 6 logs (MPN/mL) when spores were treated for 5 min. The relationship of treatment time and reduction of mold population fit a linear model with high R^2 value (0.96).

Because we had speculated that there may have been a residual acid effect when apple discs were dipped in acetic acid sanitizer and stored, we evaluated the effectiveness of treating *P. expansum*–inoculated apple discs with 2% acetic acid for various lengths of time (Table 3). A wash treatment with 2% acetic acid solution for at least 1 min completely inhibited the development of brown lesions on inoculated apple discs during storage and, consequently, no patulin was detectable after a 7-d incubation period. When the treatment time was shorter than 1 min, mold growth was observed at various levels and patulin was produced. In contrast, treatment with a 5% acetic acid solution for 1 s completely inhibited mold growth on inoculated apple discs (data not shown). Incubation temperatures of 25 °C were used throughout this study to enhance mold growth. The effect of ≥2% acetic acid treatment on apples stored at lower temperatures, such as 4 °C, must still be evaluated.

Conclusions

Our results indicate that acetic acid at concentrations of 2% to 5% could be an effective sanitizer for apples against spores of *P. expansum* when appropriate contact time is ensured and resid-
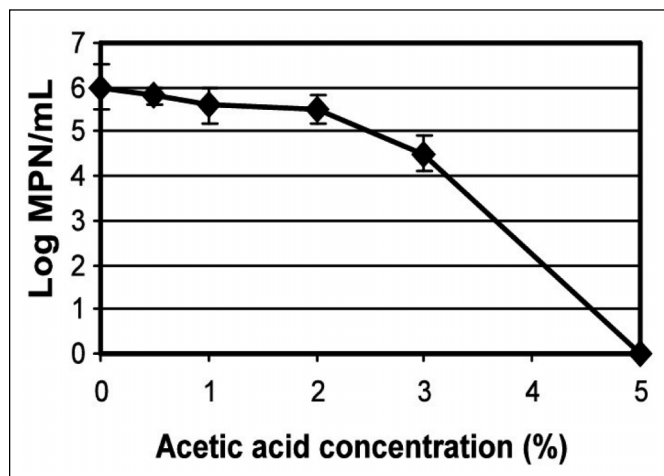
Table 2—Effect of acetic acid wash solutions of various concentrations on the growth of *Penicillium expansum* NRRL 2304 (10^3 spores originally inoculated) and patulin production on Empire apple discs treated for 5 min and then stored for 7 d at 25 °C[a]

Acetic acid concentration(%)	Mold population log (Most Probable Number [MPN]/g apple)	Patulin content (μg/g apple)
0	7.9 a	182 a
0.5	7.4 a	182 a
1	7.3 b	11 b
2	<0.7 c	0 c
3	<0.7 c	0 c
5	<0.7 c	0 c

[a]Numbers labeled with different letters indicate significant differences between treatments. Measurements are means of 2 separate trials.

Table 3—Effect of treatment time with 2% acetic acid on the growth of *Penicillium expansum* NRRL 2304 (10^3 spores originally inoculated) and patulin production on Empire apple discs during storage for 7 d at 25 °C[a]

Treatment time (s)	Mold population log (Most Probable Number [MPN]/g apple)	Patulin content (μg/g apple)
Control	7.9 a	175 a
1	7.6 a	175 a
10	6.9 b	155 a
30	3.9 c	30 b
60	< 0.7 d	0 c

[a]Numbers labeled with different letters indicate significant differences between treatments.

ual acid is not removed by rinsing from apples before storage. Treatment of apples with ≥2% acetic acid in a bin-dump tank for at least 1 min may be an effective HACCP intervention against *P. expansum*, assuming that the apples are not subsequently washed before storage. A 5% acetic acid solution, because it requires no more than 1 s of contact time, could be an effective intervention if applied from a spray nozzle on an apple sorting line as long as there is
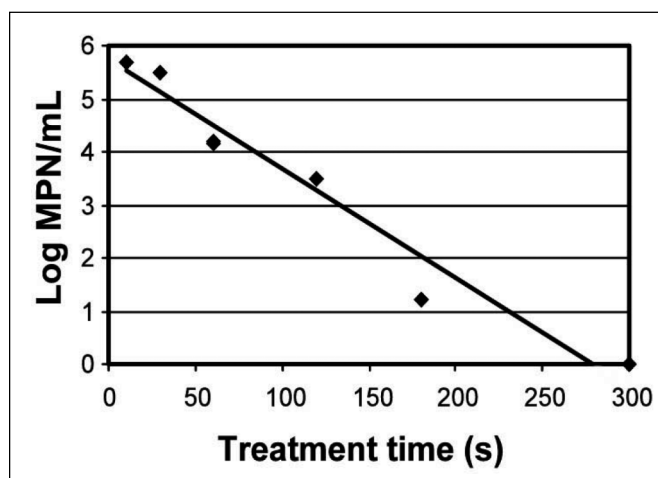

Figure 6—Survival of *Penicillium expansum* NRRL 2304 spores (10^6 Most Probable Number [MPN]/mL) after 5 min of treatment in acetic acid solutions of varying concentrations

Figure 7—Survival of *Penicillium expansum* NRRL 2304 spores (10^6 Most Probable Number [MPN]/mL) after treatment in 5% acetic acid solution for various times

no subsequent wash step before storage. Application of acid either in a bin-dump tank or as a spray solution in a sorting line would be easy to implement and could be readily adopted by both small-scale and large-scale apple processors. Our results further indicate that an intervention against *P. expansum* can effectively inhibit the production of patulin. An informal sensory analysis in our laboratory with 5% acetic acid–treated and stored Empire apples indicated that, unlike other chemical treatments, acetic acid does not affect the taste of the apples, regardless of whether or not the apples are washed after storage and before tasting (data not shown). Processors, however, routinely wash apples upon removal from storage and before sorting apples for cider. Our research, therefore, indicates that a processor could effectively treat apples with 2% to 5% acetic acid after harvest and before storage, and subsequently process the apples into juice or cider with no expected change in the flavor of the juice or cider.

The conclusions of our study are, however, predicated on our apple-slice model effectively modeling the behavior of *P. expansum*–contaminated, intact fruit. As our initial experiments aimed at developing an inoculation system suggest, sanitizer effectiveness may be limited in wounded, intact fruit by lack of sanitizer-pathogen contact. Nevertheless, because a 2% to 5% acetic acid treatment would be an easy-to-implement intervention against *P. expansum*, evaluation of this treatment in an pilot-scale apple-processing facility is warranted.

Acknowledgment

References

Bissessur J, Permaul J, Odhav B. 2001. Reduction of patulin during apple juice clarification. J Food Prot 64:1216–9.

Brackett RE, Marth EH. 1979a. Ascorbic acid and ascorbate cause disappearance of patulin from buffer solution and apple juice. J Food Prot 42:864–6.

Brackett RE, Marth EH. 1979b. Patulin in apple juice from roadside stands in Wisconsin. J Food Prot 42:862–3.

Brause AR, Trucksess MW, Thomas FS, Page SW. 1996. Determination of patulin in apple juice by liquid chromatography: collaborative study. Food Chem Contam 79:451–5.

Burda K. 1992. Incidence of patulin in apple, pear and mixed fruit products marketed in New South Wales. J Food Prot 40:796–8.

Ciegler A, Beckwith AC, Jackson LK. 1976. Teratogenicity of patulin and patulin adducts formed with cysteine. Appl Environ Microbiol 31:664–7.

Conway WS, Janisiewicz WJ, Klein JD, Sams CE. 1999. Strategy for combining heat treatment, calcium infiltration, and biological control to reduce postharvest decay of 'Gala' apples. HortSci 34:700–4.

Damoglou AP, Campbell DS. 1985. The effect of pH on the production of patulin in apple juice. Lett Appl Microbiol 2:9–11.

Doores S. 1983. The microbiology of apple and apple products. CRC Crit Rev Food Sci Nutr 19:133–49.

Eifert JD, Sanglay GC. 2002. Chemistry of chlorine sanitizers in food processing. Dairy Food Environ Sanit 22:534–8.

Gokmen V, Artik N, Acar J, Kahraman N, Poyrazoglu E. 2001. Effects of various clarification treatments on patulin, phenolic compound and organic acid compositions of apple juice. Eur Food Res Technol 213:194–9.

Harrison M. 1989. Presence and stability of patulin in apple products: a review. J Food Safety 9:147–53.

Hopkins J. 1993. The toxicological hazards of patulin. Br Ind Biol Res Assoc Bull 32:3–4.

Huebner HJ, Mayura K, Pallaroni L, Ake CL, Lemke SL, Herrera P, Phillips TD. 2000. Development and characterization of a carbon-based composite material for reducing patulin levels in apple juice. J Food Prot 63:106–10.

Kadakal C, Sebahattin N, Poyrazoglu ES. 2002. Effect of commercial processing stages of apple juice on patulin, fumaric acid and hydroxymethylfurfural (HMF) levels. J Food Qual 25:359–68.

Lang MM, Ingham SC, Ingham BH. 1999. Verifying apple cider plant sanitation and HACCP programs: Choice of indicator bacteria and testing methods. J Food Prot 62:887–93.

Lennox JE, McElroy LJ. 1984. Inhibition of growth and patulin synthesis in *Penicillium expansum* by potassium sorbate and sodium propionate in culture. Appl Environ Microbiol 48:1031–3.

Lindroth S, Niskanen A. 1978. Comparison of potential patulin hazard in homemade and commercial apple products. J Food Sci 43:446–8.

Mayer VW, Legator MS. 1969. Production of petite mutants of *Saccharomyces cerevisiae* by patulin. J Agric Food Chem 17:454–6.

McCallum JL, Tsao R, Zhou T. 2002. Factors affecting patulin production by *Penicillium expansum*. J Food Prot 65:1937–42.

Moodley RS, Goviden R, Odhav B. 2002. The effect of modified atmospheres and packaging on patulin production in apples. J Food Prot 65:867–71.

Neogen Corp. 1999. Iso-Grid® methods manual. 3rd ed. Lansing, Mich.: Neogen Corp.

Okull DO, LaBorde LF. 2004. Activity of electrolyzed oxidizing water against *Penicillium expansum* in suspension and on wounded apples. J Food Sci 69:FMS23–7.

Podgorska E. 1992. Effect of preservatives on patulin production by *Penicillium expansum*. Acta Microbiol Polonica 42:97–107.

Roberts RG, Reymond ST. 1994. Chlorine dioxide for reduction of postharvest pathogen inoculum during handling of tree fruits. Appl Environ Microbiol 60:2864–8.

Rychlik M, Schieberle P. 2001. Model studies on the diffusion behavior of the mycotoxin patulin in apples, tomatoes, and wheat bread. Eur Food Res Technol 212:274–8.

Ryu D, Holt DL. 1993. Growth inhibition of *Penicillium expansum* by several commonly used food ingredients. J Food Prot 56:862–7.

Sholberg P, Haag P, Hocking R, Bedford K. 2000. The use of vinegar vapor to reduce postharvest decay of harvested fruit. HortSci 35:898–903.

Stott WT, Bullerman LB. 1975. Patulin: a mycotoxin of potential concern in foods. J. Milk Food Technol 38:695–705.

Sydenham EW, Vismer HF, Marasas WFO, Brown NL, Schlechter M, Rheeder JP. 1997. The influence of deck storage and initial processing on patulin levels in apple juice. Food Addit Contam 14:429–34.

Sydenham EW, Vismer HF, Marasas WFO, Brown N, Schlechter M, van der Westhuizen L, Rheeder JP. 1995. Reduction of patulin in apple juice samples-influence of initial processing. Food Control 6:195–200.

[USFDA] U.S. Food and Drug Administration. 1998. Guide to minimize microbial food safety hazards for fresh fruits and vegetables. October 1998. Washington, D.C.: U.S. Food and Drug Administration. Available at: http://vm.cfsan.fda.gov/~dms/prodguid.html. Accessed 1 April 2004.

[USFDA] U.S. Food and Drug Administration. 2002a. Compliance policy guide. Sec. 510.150. Apple juice, apple juice concentrates, and apple juice products—adulteration with patulin. Issued 22 Oct 2001. Washington, D.C: U.S. Food and Drug Administration. Available at: http://www.fda.gov/ora/compliance_ref/cpg/cpgfod/cpg510-150.htm. Accessed 1 April 1 2004.

[USFDA] U.S. Food and Drug Administration. 2002b. U.S. Food and Drug Administration. Juice HACCP regulator training. September 2002. Washington, D.C: USFDA Center for Food Safety and Applied Nutrition. Office of Field Programs. Available at: http://www.cfsan.fda.gov/~comm/juiceman.html. Accessed 1 April 2004.

Venturini ME, Blanco D, Oria R. 2002. In vitro antifungal activity of several antimicrobial compounds against *Penicillium expansum.* J Food Prot 65:834–9.

Wheeler JL, Harrison MA, Koehler PE. 1987. Presence and stability of patulin in pasteurized apple cider. J Food Sci 52:479–80.

Yurdun T, Omurtag GZ, Ersoy O. 2001. Incidence of patulin in apple juices marketed in Turkey. J Food Prot 64:1851–3.

C: Food Chemistry & Toxicology