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Patulin: Surveillance in Michigan Apple Cider and Juice, and Factors Influencing Its Production and Concentration in Apple Products

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PATULIN: SURVEILLANCE IN MICHIGAN APPLE CIDER AND JUICE, AND FACTORS INFLUENCING ITS PRODUCTION AND CONCENTRATION IN APPLE PRODUCTS

BY

Kerri Latrice Harris

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Abstract

PATULIN: SURVEILLANCE IN MICHIGAN APPLE CIDER AND JUICE, AND FACTORS INFLUNENCING ITS PRODUCTION AND CONCENTRATION IN APPLE PRODUCTS

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Patulin is produced by mold species such as *Penicillium expansum* and is the most abundant mycotoxin in apples and apple juice. Because of its toxicity, the FDA has established that patulin concentrations in apple juice products should not exceed 50 μg/kg. The objectives of this research were: 1) determine the concentrations of patulin in a) apple cider produced and marketed by Michigan cider mills in 2002-2004 and b) different brands of apple juice and cider, including shelf-stable products, marketed in retail grocery stores in Michigan in 2005-2006, 2) determine the production of ethylene by *P. expansum* and the relationship between the presence of endogenous and exogenous ethylene and patulin production by *P. expansum* on liquid and solid media, 3) determine whether fruit-produced ethylene or exogenous ethylene (100 μL/L) affects the production of patulin in apples treated with 1-methylcyclopropene (1-MCP) and inoculated with *P. expansum,* and 4) determine the effect of trimming and culling of apples inoculated with *P. expansum* on patulin levels in intact and decayed apple tissue and in juice produced from two varieties of apples. To achieve these objectives, we 1) surveyed cider and juice samples from Michigan mills (N=493) retail grocery stores (N=159) for patulin concentrations, 2) analyzed the effects of ethylene and 1-MCP on patulin production in Red Delicious apples and liquid and solid media inoculated with *P. expansum*, and 3)

evaluated the effects of trimming and culling on patulin concentrations in juice from naturally-infected Red Delicious apples and manually inoculated (*P. expansum*) Jonagold and Red Delicious apples stored in normal atmosphere for up to 15 days.

The survey confirmed detectable patulin (≥4 μg/L) in 18.7% of all cider mill samples with 2.2% of samples above the legal limit of 50 μg/L. In retail grocery stores, 23% contained detectable patulin, with 11.3% of samples having patulin concentrations above the legal limit. Exogenous ethylene administration significantly promoted patulin production by *P. expansum* when inoculated on solid Potato Dextrose Agar media ($P \leq 0.0206$) and tended to promote ($P \leq 0.065$) patulin production by *P. expansum* when inoculated on apples. 1-MCP treatment of apples throughout *P. expansum* growth did not influence patulin production in either medium. Juice extracted from rotten tissue of apples inoculated with *P. expansum* in the laboratory typically contained >1000 μg patulin/L by six days after inoculation. Juice prepared from normal appearing tissue of both Jonagold and Red Delicious apple varieties always contained detectable patulin by nine days after inoculation, but patulin concentrations in this normal-appearing tissue never exceeded 100 μg/L.

These experiments demonstrated that 1) apple cider and juice processors need to improve patulin control procedures, 2) exogenous ethylene treatment does not inhibit, and may actually enhance, patulin production by *P. expansum*, and 3) that careful trimming and culling of fruit used for juice production is highly effective in controlling patulin concentrations in apple juice, provided that removal of decayed apple flesh is essentially complete.

DEDICATION

This work is dedicated to all those who loved me enough to pray for me, and those who came before me, especially my parents, Willie and Emma Harris; my sister, Kara J. Harris; my grandparents, Mary E. Hudson, Minnie C. Adams, and Theophilus Rembert.

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children. I always felt welcome in their home, even when I appeared unaccounced and asking for something. Mr. and Mrs. Karl and Connie Wilson always made themselves available when I needed help in any manner. Not only Shay, but the entire Davis Family kept me in their prayers and were only a phone call away when needed.

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Introduction

Patulin (4-hydroxy-4H-furo(3,2c)pyran-2(6H)-one) has a molecular weight of 154.12 and an empirical formula of $C_7H_6O_4$ (Woodard and Singh 1949, Woodard and Singh 1950). This crystalline, colorless compound has a melting point of 110-112°C and sublimes in high vacuum at 70-100°C (Chain et al. 1942, Katzman et al. 1944). Patulin is a secondary fungal metabolite, is the most common mycotoxin found in apples and apple juices, and is produced by several species of *Penicillium*, *Aspergillus*, and *Byssochlamys* fungi (Wilson 1974, Lindroth 1980, Martins 2002). *Penicillium expansum* is the primary source of patulin in fruit (Askar 1999, Jackson et al. 2003).

Following its discovery in 1941, patulin was initially widely studied due to its antibiotic activity against some gram-positive and gram-negative bacteria, as a possible cure for the common cold, and some skin infections, but this research was abandoned because of its toxic side effects (Raistrick et al. 1943, Martins 2002, Sorenson et al. 1985). Studies continued on the phytotoxicity and mutagenic properties of patulin (Dickens and Jones 1961, Pohland and Allen 1970, Lindroth 1980). Patulin causes gastrointestinal toxicity in rodents and also has mutagenic, neurotoxic, genotoxic and carcinogenic effects in rodents (Dickens and Jones 1961, Osswald 1978, Ciegler 1976, Reddy 1978, Reddy 1979, Lindroth 1980). Furthermore, patulin causes dermal and gastric irritation in humans and also suppresses the immune system of rodents, producing ulceration, congestion, and hemorrhagic lesions, especially in the gastrointestinal tract (Dickens and Jones 1961, McKinley et al. 1980ab, DeRosnay 1952, Dalton 1952).

Based upon this toxicity profile, an action level for patulin was established at 50 µg/kg in apple juice and in beverages containing apple juice ingredients (FDA 2001,

Codex Alimentarius Commission 2003). In recent years, patulin surveillance in apples and apple juices by regulatory authorities in the U.S. and other countries has resulted in significant product recalls due to patulin concentrations in excess of regulatory limits. Thus, patulin contamination of food products has significant economic implications for food processors in addition to its detrimental effects on public health.

The **overall objectives** of this research were to determine the prevalence and levels of patulin contamination in apple cider and juice in Michigan and to assess factors influencing patulin production in apples and its resulting concentration in apple juice products. In order to meet these overall objectives, experiments were conducted to achieve the following **specific aims**:

1. Determine the concentrations of patulin in a) apple cider produced and marketed by Michigan cider mills in 2002, 2003, and 2004; and b) different brands of apple juice and cider, including shelf-stable products, marketed in retail grocery stores in Michigan in 2005 and 2006.
2. Determine the production of ethylene by *Penicillium expansum* and the relationship between the presence of endogenous and exogenous ethylene and patulin production by *Penicillium expansum* on liquid and solid media.
3. Determine whether fruit-produced ethylene or exogenous ethylene (100 μL/L) affects the production of patulin in apples treated with 1-Methylcyclopropene (1-MCP) and inoculated with *Penicillium expansum*.
4. Determine the effect of trimming and culling of apples inoculated with *Penicillium expansum* on patulin levels in intact and decayed apple tissue and in juice produced from two varieties of apples.

The **expected outcomes** of this project are that the apple juice and cider industry will have greater awareness of potential for patulin contamination in their products and will have improved methods to reduce or prevent patulin contamination in apple and apple juice products. We hope that increased awareness of patulin as a hazard in apples and processed apple products will enable the industry to take preventative actions which will lead to cost savings and a decreased risk of product recalls and adverse publicity.

1 Patulin: Review of the Literature

1.1 Rationale and Public Health Significance

Patulin is the most common mycotoxin found in apples and apple juices (Essa and Ayesh 2000) and is produced by several species of *Penicillium*, *Aspergillus*, and *Byssochlamys* fungi. *Penicillium expansum* is the main cause of apple rot and the primary source of patulin formation in fruit (Ruggieri 1982, Askar 1999, Jackson 2003). Due to concerns over human safety, the World Health Organization (WHO) recommended that the maximum allowable concentration of patulin in apple juice be 50 µg/L (Van Egmond 1989, Stoloff, et. al. 1991). The Codex Alimentarius Commission (2003) standard recommends an action limit for patulin of 50 µg/kg in apple juice and in beverages containing apple juice ingredients. The regulatory limit in the United States for apple products is no more than 50 µg/kg of patulin in apple juice, apple juice concentrate (when diluted to single strength), and applesauce (FDA 2001). Patulin regulations were needed because small amounts of *P. expansum* contamination of apples can lead to high patulin concentrations in processed apple products depending upon the isolate, temperature, storage time, and apple variety (McCallum et al. 2002), and because patulin is a chemical hazard to human health.

Patulin has mutagenic, neurotoxic, genotoxic, carcinogenic and gastrointestinal toxicity effects in rodents. It also causes dermal and gastric irritation in humans (Dickens and Jones 1961, Sydenham et. al. 1997, Moodley 2002). Patulin has antifungal activity and is extremely toxic to plant and animal cells (Sorenson et. al. 1985, Martins 2002). Patulin also suppresses the immune system of rodents and produces ulceration,

congestion, and hemorrhagic lesions, especially in the gastrointestinal tract (McKinley et al. 1980ab, Martins et al. 2002).

Michigan is one of the leading states in production of apples and apple products in the United States, producing and processing an average of 762 million pounds of apples in 2001-2005 (NASS 2004-2005). An average of 172 million pounds (23%) of all apples produced in Michigan were processed to juice and cider in 2001-2005 (NASS 2004-2005).

Mold growth in fruits results in significant economic losses worldwide (Ryu and Holt 1993). Blue mold, the disease caused by *Penicillium sp.* in apples, leads to soft, watery, brown spots that quickly enlarge, especially when fruit is stored at moderate temperatures. These spots generally appear after some sort of damage or bruising has occurred to the fruit, thereby allowing a point for fungal invasion and growth (Moodley 2002). Among the molds that commonly colonize apples, *P. expansum* is the major source of patulin (Harwig et al. 1973, Brian et al. 1956).

Patulin surveillance in apple juices produced by Michigan cider mills is important to determine the incidence and levels of patulin contamination in apple cider and juice throughout the state. These data can be used as an indicator of the quality of apples used to make these products and to predict the prevalence of human consumption of products containing patulin concentrations above the legal limit of 50 μg/kg (μg/L).

The effects of ethylene on patulin production in stored, 1-MCP treated apples have not been investigated. Although ethylene promotes ripening of fruit, at it is also possible that ethylene may prevent the development of patulin in apple products that are eventually pressed and used for juices and ciders.

Although previous studies on the effects of trimming and culling on patulin concentrations in apples have been conducted, no systematic and quantitative trimming studies have been determined. Trimming may be as effective as culling to reduce patulin contamination, depending on the initial amount of patulin in the product.

1.2 Incidence in Food and Beverage Products

Patulin is the most common mycotoxin found in apples and apple juices (Essa and Ayesh 2000). In fact, it has been found in at least 50% of apples and pears with brown rot (Frank 1977). Patulin is formed from many mold species and found in several food items - bread, pears, peaches, plums, apricots, bananas, cherries, pineapples, grapes, moldy silage, and was temporarily found in fermented sausage during the ripening process (Reiss 1972, Tyllinen et al. 1977, Anderson et al. 1978, Frank et al. 1977, Escoula 1975). Furthermore, patulin has been found in processed fruit products such as juices, sauces, purees, jellies, diced apples, and apple pulps (Burda 1992). Homemade apple juice and jam have been found to be at great risk for patulin contamination due to poor raw material selection and storage conditions (Lindroth and Niskanen 1978).

Inoculation studies with *P. expansum*, *B. nivea*, and *P. urticae* show that patulin can be produced in greengages, strawberries, honeydew melons, tomatoes, cucumbers, carrots, and red and green paprika (Frank 1977). However, this inoculated growth failed in celeriac, kohlrabi, cauliflower, red cabbage, radish, horseradish, onions, zucchini squash, potatoes, and egg plants (Frank 1977).

It should be noted, however, that low-carbohydrate and high-protein foods generally do not support the production of significant amounts of patulin even though such foods may have extensive mold growth (Olivigni and Bullerman 1977, Stott and

Bullerman 1975). The presence of and reaction with sulfhydryl groups present in high-protein foods makes patulin chemically undetectable and less toxic (Olivigni and Bullerman 1977, Stott and Bullerman 1975).

1.3 Patulin Biosynthesis

Patulin (4-hydroxy-4H-furo(3,2c)pyran-2(6H)-one) has a molecular weight of 154.12 and an empirical formula of $C_7H_6O_4$ (Woodard and Singh 1949, Woodard and Singh 1950). This secondary metabolite is produced by several species of *Penicillium*, *Aspergillus*, and *Byssochlamys* fungi, namely, *P. expansum* (*P. leucopus*), *P. urticae* (*P. patlum/P. griseofulvum*), *P. claviforme, P. cyclopium, P. equinum, P. granulatum* (*P. divergens*), *P. lanosum, P. lapidosum, P. melinii, P. novae-zeelandiae, P. roqueforti, A. clavatus, A. giganteus, A. terreus, B. fulva* and *B. nivea* (Lindroth 1980, Paster 1995, Brause 1996, Martins 2002, Sydenham et. al 1997, Harrison 1989, Northolt 1978, Berretta et al. 2000, Davis and Diener 1978, Burda 1992, Gokmen 2001, Taniwaki et al. 1989, Roland 1984, Essa and Ayesh 2000, Harwig 1978, Rice et al. 1977, Wilson 1974).

Like most mycotoxins, little is known about the biosynthesis of patulin. Mycotoxins are secondary metabolites of fungi that are formed at the end of the exponential growth phase of the fungus (Maggon 1977). The most agreed upon theory is that these secondary metabolites are formed when there is an accumulation of primary metabolic precursors, such as acetate, malonate, pyruvate, and amino acids, due to slowed organism growth and reduced energy requirements. The fungus tries to efficiently rid itself of these precursors by diverting them to secondary metabolites (Maggon 1977). Many of these metabolites are precursors in fatty acid synthesis that alternately follow a polyketide pathway since the additional energy will not be used

(Scott et al. 1973, Tanenbaum and Bassett 1959). This polyketide pathway leads to the biosynthesis of 6-methylsalicyclic acid (6-MSA), the first precursor to patulin and many other mycotoxins (Scott et al. 1973, Tanenbaum and Bassett 1959, Zamir 1980). It is thought acetyl-CoA and Malonyl-CoA combine and the enzyme 6-MSA synthase is introduced to create 6-MSA (Scott et al. 1973, Zamir 1980). After several more steps, gentisaldehyde is formed, followed by patulin (Scott et al. 1973, Zamir 1980).

1.4 Patulin Production and Stability

Numerous studies have been conducted on factors affecting patulin production by *Penicillium* species. *Penicillium expansum* is the most common species studied since it has been found in a wide variety of foods such as fruits, vegetables, bread, and meat products. This wide distribution of *P. expansum* in food products is probably due to its tolerance of low pH and its stability over a wide range of temperatures including normal pasteurization times and temperatures (Rychlik 2001, McCallum et al. 2002). *Penicillium* can grow and produce patulin in apples at temperatures below 5°C in normal atmospheric storage (Northolt et al. 1978, Northolt and Bullerman 1982, Sydenham et al. 1997). *P. expansum* is especially noted for the production of patulin in apples and apple products (Pohland et al. 1970, Davis and Diener 1978, Sydenham et al 1997). The presence or absence of patulin cannot be determined based solely upon the visible presence or absence of mold, since toxins may remain long after molds disappear (Lindroth 1980, Sydenham et al. 1997, Jackson 2003). Sound apples inoculated with *P. expansum* showed that the initial firmness of the apple and the cultivar of apple might be better determinants of the amount of patulin produced, rather than visibility of rot (Beer and Amand 1974).

Patulin production is highly dependent upon several factors including water activity (A_w), preharvest treatment, the type of food product and the type of fungal species and strains present (Northolt et al. 1978). Because of the narrow A_w range (0.95-0.99) for patulin production, water activity is an important determinant of patulin production by *P. expansum* and *P. patulum* (Northolt et al. 1978, Martins et al. 2002). Preharvest handling and treatment of apples can influence patulin production in apples (Watkins et al. 1990, Acar et al. 1998, Jackson 2003). Dropped or windfall apples are more likely to be contaminated with patulin than tree-picked apples. Unculled tree-picked fruit is more likely to have patulin contamination than culled fruit (Jackson 2003).

Little or no patulin usually penetrates healthy tissue surrounding the disease lesions unless fruits are overripe or had senescent breakdown (Sydenham et al. 1997, Jackson 2003). However, patulin may be present in visibly healthy fruit (Sydenham et al. 1997, Jackson 2003, Lindroth 1980). Patulin has been known to be present up to 1-2 cm from the source of visible rot in apples (Taniwaki et al. 1989, Sydenham et al. 1997, Rychlik 2001, Moodley et al. 2002). However, patulin can penetrate an entire tomato (Rychlik 2001). Juices with pulp are more susceptible to patulin contamination than other plain juice products and fruit by-products (Beretta 2000, Buchanan et al. 1974).

Once formed, patulin is completely stable in apple juice, grape juice and dry corn. Traditional apple processing methods will not eliminate patulin in the final products (Pohland and Allen 1970, Scott and Somers 1970). Patulin is unstable in alkaline pH (Chain et al. 1942, Raistrick 1943, Karow 1944, Heatley et al. 1947). In fact, it is slowly degraded at pH 6.8 (Brackett and Marth 1980).

1.5 Patulin Toxicity

1.5.1 Humans

Initial studies that claimed patulin may be a cure for the common cold could not be validated (Medical Research Council 1944, Stoloff 1976). Patulin is of utmost concern to humans not only due to its acute toxicity, but also because of its suspected carcinogenic and teratogenic properties (Dickens and Jones 1961, Martins et al. 1982, Martins 2002). Patulin can cause extensive tissue swelling as well as additional epidermal and dermal injury when applied directly to the skin of humans (Dalton 1952). Patulin also causes acute toxicity when ingested (Dickens 1965, Martins 2002.) Patulin was detected in frozen blueberries and was suspected of causing diarrhea in the children who ate them (Lindroth 1980b). In humans, oral administration of patulin (100 mg over 10 days) results in stomach irritation, nausea, vomiting, and diarrhea (Walker and Wiesner 1944). However, intravenous perfusion of 100 mg of patulin into a human caused no acute toxicity (DeRosnay 1952).

1.5.2 Animals

Acute toxicity and death caused by patulin has been assessed using a variety of animal models. Table 1 summarizes the LD_{50} for patulin observed in several studies. The LD_{50} by daily oral administration of patulin to mice for two weeks is 29 to 48 mg/kg body weight, and 125 mg/kg body weight was always fatal (Broom et al. 1944, Lindroth and von Wright 1978, McKinley and Carlton 1980ab). Seventy-five mg/kg body weight of patulin was always fatal in mice and rats when injected intravenously (Broom et al. 1944, Chain et al. 1942, Raistrick et al. 1943, Yamamoto 1954). Comparable results have been produced in rats, cats, dogs, hamsters, cows, and rabbits following oral

administration of patulin (Broom et al. 1944, Lindroth and von Wright 1978, McKinley and Carlton 1980ab). Subcutaneous, intravenous and intraperitoneal injection of patulin in mice, rats, hamsters, and dogs all resulted in generally similar LD_{10} values, ranging from 5.7 - 50 mg patulin/kg body weight in these species. Chickens and their embryos, quail, rabbit skin, guppies, and zebra fish larvae also were found to be sensitive to patulin (Broom et al. 1944, McKinley and Carlton 1980ab, Ciegler et al. 1977). Animals that did not die during these acute toxicity studies demonstrated severe pathological changes, such as hemorrhaging in the lungs, capillary damage in the liver, spleen and kidney, and edema of the brain (Broom et al. 1944, Lindroth and von Wright 1978, McKinley and Carlton 1980).

In a long-term rodent toxicity study by Dickens and Jones (1961), it was found that patulin should be considered a carcinogen. Male rats were subcutaneously injected with 0.2 mg of patulin in arachis oil two times per week for 61-64 weeks (Dickens and Jones 1961). Local sarcomas were observed in six out of eight surviving rats (Dickens and Jones 1961). However, no carcinogenic activity attributable to patulin was observed in an experiment where patulin was administered orally by a stomach tube to female Sprague-Dawley rats two times per week for 64 weeks with a total dosage of 358 mg of patulin/kg body weight (Osswald et al. 1978). Becci et al. (1981) also conducted a long-term study of 109 weeks. Male and female rats were administered 0.0, 0.1, 0.5, and 1.5 mg/kg body weight of patulin by gastric intubation three days a week. It was found that repeated oral administration of 1.5 mg/kg body weight of patulin caused premature death in rats (Becci et al. 1981). No adverse effects were noted at 0.1 mg/kg body weight of

patulin. The information from this study was used by FDA to develop the maximum residue limits (MRL) for patulin (FDA 2001).

Table 1. Summary of Patulin LD_{50} values in animals (from Lindroth 1980)

Animal	Administration*	LD_{50} (mg/kg)	Reference
Mouse	Sc	8-10	Katzman et al. 1944
	Sc	10.0	McKinley and Carlton 1980b
	Sc	15	Broom et al. 1944
	Iv	15.6	Yamamoto 1954
	Iv	25	Broom et al. 1944
	Ip	5.7	Ciegler et al. 1976
	Ip	7.5	McKinley and Carlton 1980b
	Ip	15	Hofmann et al. 1971
	Ip	15	Broom et al. 1944
	Ip	30	Andraud et al. 1964
	Po	29	Lindroth and von Wright 1978
	Po	35	Broom et al. 1944
	Po	48	McKinley and Carlton 1980b
Rat	Sc	15	Broom et al. 1944
	Sc	25	Katzman et al. 1944
	Iv	25-50	Broom et al. 1944
Hamster	Sc	23	McKinley and Carlton 1980a
	Ip	10	McKinley and Carlton 1980a
	Po	31.5	McKinley and Carlton 1980a
Dog	Iv	10.4	Reddy et al. 1979
Chick	Po	170	Lovett 1972
Chicken embryo (4-day-old)		2.35 µg/embryo	Ciegler et al. 1976
Zebra fish larvae		18.0 µg/ml	Abedi and Scott 1969

*sc subcutaneous injection
iv intravenous injection
ip intraperitoneal injection
po per os

1.5.3 Plants

Patulin inhibits, wilts, or stunts the growth of the seeds of cucumbers, corn, peas, flax, safflower seedlings, and sugar beets (Berestets'kyi and Synyts'kyi 1973, Gattani, ML 1957, Wallen and Skolko 1951, Lindroth 1980). Furthermore, patulin inhibits

germination of apple pollen and the growth of cultured soybean cell suspensions, tomato seedlings, and wheat shoot (Polacco and Sands 1977, Norstadt and McCalla 1963, Miescher 1950, Lindroth 1980). Patulin reduces wheat straw and grain yields in greenhouse and growth chamber cultivation conditions (Ellis et al. 1977, Norstadt and McCalla 1971, Lindroth 1980). Field studies confirmed that patulin decreases germination, plant growth, winter survival and tillering of wheat seedlings and reduces grain yield (Ellis et al. 1973)

1.6 Detection

Several methods, including thin-layer chromatography, gas chromatography, gas chromatography/mass spectrometry, fluorodensitometric assay, and liquid chromatography/mass fragmentography, have been successfully used in the determination of patulin (Salem and Swanson 1976, MacDonald et al. 2000, Lindroth 1980). A summary of these methods and their limits of detection for patulin is presented in Table 2. High performance liquid chromatography is presently acknowledged as the standard method by the Association of Official Analytical Chemists (AOAC; MacDonald et al. 2000). The AOAC method involves extraction and isolation of patulin followed by its separation by reverse phase liquid chromatography and detection by ultraviolet light absorbance (MacDonald et al. 2000).

Several methods of patulin extraction prior to liquid chromatography have been developed. The two most common methods are extraction into ethyl acetate followed by back-extraction into sodium carbonate, or extraction into ethyl acetate with cleanup on a silica gel column (MacDonald et al. 2000). Another method utilizes mixtures of acetonitrile with water or 4% aqueous KCl (9:1) to extract patulin from apples and pears

and their by-products (Gimeno and Martins 1983). The detection limit of patulin in foods using HPLC with UV detection is approximately 5 μg/kg (Woodward and Singh 1949, Pohland and Allen 1970). Patulin's single ultraviolet (UV) absorption maximum is at approximately 275 nm (Pohland and Allen 1970, Woodward and Singh 1949). Eisle and Gibson (2003) developed a syringe-cartridge solid phase extraction method for patulin in apple juice that has a sample recovery average greater than 92%.

Table 2. Synopsis of Different Methods of Detection of Patulin

Type of Instrument	Limit of Detection	Type of Product	Authors
HPLC	1-11 µg/kg	Apple Juices and Butter	Ware 1975, Ware et al. 1974, Scott and Kennedy 1973, Tanner and Zanier 1976, Stray 1978
	5 µg/kg	Breakfast cereals, pork, baked beans, cheese, milk powder	Hunt et al. 1978
	5 µg/kg	Fruit Juices	Leuenberger et al. 1978
	2.7 µg/kg	Apple Juice	MacDonald et al. 2000
TLC	40 µg/kg	Corn	Pohland and Allen 1970, Scott 1974, Scott and Kennedy 1973
With 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH)	20 µg/l	Apple Juice	AOAC 1975, Abedi and Scott 1969
Reflectance measurement at 273 nm	40 µg/kg	Apples, pears, tomatoes, cucumbers, plums	Polzhofer 1977
With Diethylamine	20 µg/kg	Cheese	Siriqadana and Lafont 1979
GLC	20 µg/kg	Grains	Fujimoto et al. 1975, Suzuki et al. 1975
	2.5 µg/kg	Apple Juice	Josefsson and Andersson1976, Fujimoto et al. 1975, Anderson et al. 1978
Mass Fragmentography	5 µg/kg	Apple Juice	Rosen and Pareles 1974
Fluorodensitometric Assay	10 µg/kg	Fruits and Fruit Products	Fritz et al. 1979
	0.1 µg of	Pure Patulin	Salem and Swanson 1976
	1.0 mg/l	Apple Juice	Salem and Swanson 1976
Rapid Thin Layer Chromatography	120-130 µg/kg	Apples, Pears, Apple Juice and Jam, Pear Juice and Jam	Gimeno and Martins 1983

1.7 Patulin Surveillance in Apple Juice

The presence of patulin in commercial apple products is of great concern to the apple industry. Until recently, patulin surveillance in Michigan apple juice and cider has been negligible due to a lack of industry awareness of the problem and the high costs and poor availability of patulin testing by commercial laboratories. Only recently has patulin received regulatory attention in the United States, which has increased the demand for patulin analyses. Few food laboratories offer patulin analysis and those who do may charge $100-$300 per sample. This regulatory attention, plus additional awareness and surveillance, should ultimately result in lower patulin incidence as was observed in a 1998 United Kingdom study (MAFF 1998). This study showed that the percentage of apple juice samples that contained patulin in excess of 50 µg/L decreased from 26% to 2% over a six-year period following the establishment of the action level in the UK (MAFF 1998).

Although patulin surveillance had not been previously reported for Michigan apple juice or cider, several surveillance studies have been reported for apple juice, apple juice concentrate, and related products in other regions. Table 3 summarizes several of these patulin surveillance studies.

Collectively, these studies confirm that patulin contamination of apple juice products is a significant concern worldwide. Patulin levels as high as 1,000 µg/L were present in samples of Canadian apple cider (Scott et al. 1972). The U.S. Food and Drug Administration surveyed patulin in apple juice in the U.S. market in 1973, and patulin was detected in 37% of 136 samples with an average contamination level of 69 µg/L (40-440 µg/L range) (Stoloff 1976). In Washington, D.C. area stores, 8 out of 13 apple juices

contained patulin with concentrations of 44-309 μg/L (Ware et al. 1975). On roadside stands in Wisconsin, patulin was found in 29 out of 66 samples of apple juice (Brackett and Marth 1979). Twenty-nine percent of apple juice concentrates examined in Finland contained detectable patulin with an average patulin concentration of 196 μg/L (Lindroth 1980). The same study reported that 26% of commercial apple juices contained patulin, with concentrations averaging 15 μg/L, and 12 out of 39 homemade apple juice samples contained patulin. Of these 12 patulin positive homemade apple juice samples, 9 had 68 μg/L or less and the remaining 3 samples had thousands of micrograms of patulin per liter (Lindroth 1980).

In a recent Japanese survey including domestic and imported apple juice products, 3 of 143 domestic juice products (6-10 μg/L) and 6 of 45 imported juice products (6-15 μg/L) tested positive for patulin (Watanabe and Shimizu 2005). However, all positive samples contained less than 50 μg/L patulin (Watanabe and Shimizu 2005). A survey of patulin in Iranian apple juice (N=42) and apple juice concentrates (N=23) reported 69% and 78% incidence of patulin contamination, respectively (Cheraghali et al. 2005). Sixty-nine percent of apple juice samples contained detectable (>15 μg/L) levels of patulin, while 78% of apple juice concentrates contained detectable (>15 μg/L) levels of patulin. Thirty-three percent of the apple juice samples and 56% of the apple juice concentrates were above 50 μg/L with maximum levels of 285 μg/L and 148 μg/L, respectively (Cheraghali et al. 2005). A survey of 754 retail apple juice products in Poland revealed 166 were contaminated with patulin, 8 of which exceeded 50 μg patulin/L (Szymczyk et al. 2004). A survey of patulin in 113 samples of Australian apple juice showed patulin detected in 65% of the samples (N=75), with 33 of the positive samples had levels

exceeding 50 μg/L (Watkins et al. 1990). Among these 33 samples, 8 had patulin levels greater than 300 μg/L with a maximum of 629 μg/L (Watkins et al. 1990). A survey of patulin in 100 commercial apple juices and 12 children's apple foods in Madrid, Spain showed patulin detected in 82% of the apple juices; 75% of which had levels below 10 μg/L with a maximum concentration of 170 μg/L (Prieta et al. 1994). The children's apple foods had undetectable patulin levels (Prieta et al 1994). Another South African survey of 143 apple juice samples found detectable in 24%; 5% of which were above 50 μg/L (Odhav et al. 2001).

A long-term survey was conducted on patulin in 482 Turkish apple juice concentrates, diluted to single strength, collected during 1996-1999 (Gokmen and Acar 2000). Average patulin contamination levels were 63, 43, 19, and 31 μg/L in successive years (Gokmen and Acar 2000). The percentage of concentrates above 50 μg/L decreased each respective year (48%, 34%, 8% and 8%) (Gokmen and Acar 2000). In a separate survey of 215 products in 1996-1997, Gokman and Acar (1998) found that 46% of commercial apple juice concentrates that contained patulin were above 50 μg/L. The patulin in this study ranged from 7-367 μg/L (Gokman and Acar 1998). This decrease in succeeding years may have been due to changing climatic conditions through the years and the increased use of activated charcoal during clarification of heavily decayed apples used to manufacture concentrate. A survey of patulin in 105 apple juice samples in Taipei, Taiwan found 12 samples contained patulin, all of which were below 50 μg/L (Lai et al. 2000). A survey of 20 New Zealand apple juice samples revealed 3 contaminated samples with patulin levels of 106, 133 and 216 μg/L (Wilson 1981).

An Italian survey of 40 commercial apple products, juice, vinegar, sauce, and purees, found 11 positive samples with patulin concentrations ranging from 1.4 to 74.2 μg/l (Ritieni 2003). Ten of the 40 samples were baby foods, two of which tested positive for patulin (Ritieni 2003). Both were labeled as "organic food" (Ritieni 2003). A survey of patulin in 28 local and imported apple fruits in Cairo showed that patulin levels were within the legal limits of 50 μg/L (Ayesh and Foaad 2001). A survey of 31 apple samples in South Africa revealed 8 patulin positive samples, with a range of 5 to 45 μg/L (Leggott and Shephard 2001). Six of 10 baby fruit juices and purees contained detectable patulin levels that ranged from 5 to 20 μg/L (Leggott and Shephard 2001).

The majority of commercial products contained less than 10 μg/kg of patulin; however patulin levels as high as 42 μg/kg have also been measured in these products (Geipel et al. 1981). Homemade apple juice and jam are more at risk for patulin contamination possibly due to poorer raw material selection and storage conditions (Lindroth and Niskane 1978).

Table 3. Worldwide Patulin Surveillance in Apple Juice, Concentrates and Processed Products

Country	Product	Samples Containing Detectable Patulin Percentage (%)	Samples Containing Detectable Patulin Fraction	Mean Patulin Concentration (ug/l) Among Samples Containing Detectable Patulin	Patulin Concentration Range (ug/l) Among Samples Containing Detectable Patulin	Percentage of Patulin Positve Products with Patulin Concentration ≥ 50 ug/L	Reference
Australia	Apple Juice	66	74/113		5-629	45%	Watkins et al. 1990
Canada	Apple Cider				1000 or less		Scott et al. 1972
Finland	Apple Juice Commercial	26	20/76	15	39 or less		Lindroth and Niskane 1978
Finland	Homemade Apple Juice	31	12/39		14400 or less		Lindroth and Niskane 1978
Iran	Apple Juice	69	29/42	48.1	285 or less	33%	Cheraghali et al. 2005
New Zealand	Apple Juice	15	3/20	152	106-216	15%	Wilson 1981
Poland	Retail Apple Juice	22	166/754			1.1%	Szymczyk et al. 2004
Spain	Apple Juice	82	82/100	13.8	10-170	7%	Prieta et al. 1994
Spain	Apple Juice	ND	0/12	ND	ND	ND	Prieta et al. 1994
South Africa	Apple Juice	15	2/13	7.5	5-10	0%	Leggott and Shephard 2001
South Africa	Apple Juice	24	34/143			5%	Odhav et al. 2001

Table 3. Worldwide Patulin Surveillance in Apple Juice, Concentrates and Processed Products (Continued)

South Africa	Infant Apple Juices	67	2/3	5	5	0	Leggott and Shephard 2001
South Africa	Carbonated Apple Juice	50	2/4	25	5-45	0	Leggott and Shephard 2001
South Africa	Alcoholic Apple Ciders	25	2/8	7.5	5-10	0	Leggott and Shephard 2001
Taiwan	Apple Juice	11	12/105		15-40	0	Lai et al. 2000
United States	Apple Juice	37	50/136	69	40-440		Stoloff 1976
United States (Washington, D. C.)	Apple Juice	62	8/13		44-309		Ware et al. 1975
United States (Wisconsin)	Apple Juice	44	29/66	51	10-350	29	Brackett and Marth 1979
Finland	Apple Juice Concentrates	29	26/89	196	5-1478		Lindroth and Niskane 1978
Iran	Apple Juice Concentrates	78	18/23	61.7	148 or less	56%	Cheraghali et al. 2005
Japan	Imported apple juice concentrate	13	6/45	11.5	6-15	0	Watanabe and Shimizu 2005
Turkey 1996 1997 1998 1999	Apple Juice Concentrates		482 /234 /119 /67 /62	 63 43 19 31	 5-376 8-153 < 5-103 < 5-119	 48% 34% 8% 8%	Gokmen and Acar 2000

Table 3. Worldwide Patulin Surveillance in Apple Juice, Concentrates and Processed Products (Continued)

Turkey	Apple Juice Concentrates		/215		7-367	46%	Gokmen and Acar 1998
South Africa	Whole Apple Fruit	33	2/6	10	10	0	Leggott and Shephard 2001
Italy	Commercial Apple Products	28	11/40	26.7	1.4-74.2	5%	Ritieni 2003
Italy	Baby commercial apple products	20	2/10	15.4	13.1-17.7		Ritieni 2003
South Africa	Infant Apple Purees	57	4/7	5-20	ND	0	Leggott and Shephard 2001
South Africa	Mixed fruit Juice	33	2/6	5	5 or less	0	Leggott and Shephard 2001
Japan	Domestic Apple and Mixed Juices	2.1	3/143	8	6-10	0	Watanabe and Shimizu 2005

*ND = not detected (<5 µg/kg)

1.8 Patulin Development in Stored Apples

1.8.1 Recommended Typical Apple Storage Conditions

Controlled or modified atmosphere refrigerated storage of apples is an effective method to maintain high fruit quality even after several months of storage. Apples are typically stored under the following conditions, depending on cultivar: 90-95% relative humidity, 0.5 – 8% carbon dioxide and 1 – 3% oxygen at temperatures ranging from -1 - 4°C for 1-12 months (Reed 2003). Red Delicious apples may be stored 7 to 9 months in 2.5% O_2, 2.5% CO_2, and 0°C (DeEll 2003). Empire apples may be stored 6 to 8 months in 1.5% O_2, 1.5% CO_2, and 1-2°C (DeEll 2003). Controlled atmosphere storage and more effective fruit management have increased the potential to store apples for periods up to 13 months (Doores 1983-1984, Saftner 2002). Ethylene production by apples in refrigerated storage is reduced more by controlled-atmosphere than in regular atmosphere storage (Safter 2002).

1.8.2 Controlled and Modified Atmosphere Storage and Patulin Production

Controlled or modified atmosphere storage has been shown to reduce the amount of patulin produced in stored apples. A summary of these studies is provided in Table 4. Paster et al. (1995) observed that the ability of three strains of *P. expansum* (NRRL 2034, NRRL 6069, and CBS 481.84) to grow and produce patulin in pears and apples differed under varying temperatures (0, 3, 6, 17, and 25°C). However, the maximum patulin production in apples occurred at 17°C. They also found that patulin production was inhibited when the fungi were grown in apples stored under 3%CO_2/2%O_2 (25°C). Patulin production is inhibited by storage in 3% CO_2/2% O_2. Although these conditions

are typical for pear and apple storage to delay senescence and to curb postharvest decay, *P. expansum* may still grow and produce patulin after 3-6 months under controlled atmosphere conditions (Lovett et al. 1975). *P. expansum* 1071 produced detectable patulin (0.4 to 0.6 μg/ml) under atmospheric storage conditions of 1% CO_2, 3% O_2 and 96% N and a temperature of 33°F when stored for 3 to 6 months (Lovett et al. 1975). Frank (1977) found that any increase in oxygen concentration may lead to the formation of patulin by some fungal strains in apples inoculated with *P. expansum* and stored for 5 months at 4°C, followed by being held for 2-3 days at room temperature (Frank 1977).

In a study conducted by Jackson et al. (2003), MacIntosh, Red Delicious, Golden Delicious, Gala, Fuji, Red Rome, and Granny Smith apple cultivars were stored in controlled atmosphere for 4-6 weeks at 0-2°C. In this study, patulin was not detected in cider produced from culled (removed), tree-picked apples, but patulin was detected in cider produced from unculled, tree-picked apples (concentrations ranging from 0.97 to 64 μg/L) (Jackson et al. 2003). Cider made from tree-picked, controlled-atmosphere stored apples that were culled before pressing contained 0 to 15 μg patulin/L, while cider made from unculled fruit contained 59.9 to 120.5 μg patulin/L (Jackson et al. 2003). Taniwaki et al. (1989) observed patulin production ranges of 150-311 μg/L and 100 to 300 μg/L in apples stored at 25°C and 4°C, respectively one month after inoculation with *P. expansum*.

1.8.3 Ethylene and Patulin Production

Ethylene (C_2H_4) is a natural, gaseous compound that functions as a plant hormone (Zeringue et al. 1990). It has been approved for use in agriculture for seventy-five years and is ideal for use in food systems since it is relatively safe, inexpensive, and has no

deleterious effects on the nutritional value of food and feed (Roze et al. 2004). Furthermore, ethylene is a regulatory agent in the formation of secondary metabolites (Sharma et al. 1985).

Fruit produce ethylene as a natural part of the ripening process (Doores 1983-1984). The effects of ethylene on sensory and quality aspects of fruit, particularly firmness, have been studied. Liu and Long-Jun (1986) showed that apple softening was caused by 500 μl/L ethylene in apples stored in 3% O_2 plus 3% or 5% CO_2. However, reducing O_2 concentrations during apple storage diminished this effect (Liu and Long-Jun 1986). Ethylene had little or no effect on fruit firmness at 1% O_2 and 3% CO_2 (Liu and Long-Jun 1986). With low levels of ethylene (<1 μL/L), reduced O_2 concentrations did not influence fruit firmness (Liu and Long-Jun 1986). The rate of ethylene production by the fruit, however, was reduced by lowering O_2 concentrations (Liu and Long-Jun 1986).

Fruit was found to be firmer when exposed to lower levels (<0.1 μL/L) of ethylene versus high levels (>160 μL/L) (Johnson et al. 1993). Although no effect was found on rotting of uninoculated fruit, inoculated fruit in a low ethylene environment had higher rotting incidence compared to the higher ethylene environment (Johnson et al. 1993). Red Delicious, Golden Delicious, Idared, and "orchard" apples showed little difference in firmness and ripeness when stored at ethylene concentrations of 1-3.8 ppm, 10 ppm, and 500 ppm (Liu 1977). McIntosh apples were higher in acid, had firmer texture, and were less ripe when stored at low ethylene concentrations than at 10 ppm and 500 ppm (Liu 1977). Delicious apples were stored 7 months and had severe rot in controlled atmosphere with 10 and 500 ppm ethylene, but no rot was observed with low ethylene (Liu 1977).

Ethylene has been shown to accelerate the softening of apples that can lead to rotting (Johnston et al. 2002). Non-treated Granny Smith apples softened slower at 20°C than those treated with ethylene or cold treatment, while Pacific Rose softening did not speed up after ethylene or cold treatment (Johnston et al. 2002). Ripening was increased by a two- to three-fold increase in ethylene concentration when compared to non-treated fruit at 20°C (Johnston et al. 2002). Preclimacteric McIntosh apples were studied under low (~6 ppm) and high (~1570 ppm) ethylene levels at 3.3°C for 189 days (Forsyth et al. 1969). Apples stored under low ethylene concentrations had higher firmness compared to apples stored at high ethylene levels, and this higher firmness continued for more than 7 days at room temperature (Forsyth et al. 1969). However, pH was lowered and soluble solids content was slightly increased in apples stored at low ethylene concentrations (Forsyth et al. 1969). Low ethylene treatment causes lower ethylene emission by fruit and reduced incidence of core browning (Forsyth et al. 1969).

1.8.3.1 Ethylene Effects on Fungal Metabolism

Aspergillus parasiticus and *Aspergillus nidulans* produce ethylene during the early growth phase on media, and the onset of aflatoxin biosynthesis is marked by the absence of ethylene evolution (Sharma et al. 1985). Sharma et al. (1985) found that ethylene inhibited *Aspergillus* development and toxin biosynthesis by adding 2-chloroethyl phosphonic acid (CEPA), an ethylene-generating compound, directly to growth medium. These experiments reported a tight correlation between ethylene presence and aflatoxin suppression in *A. parasiticus*, as well as between ethylene concentration and stimulation or inhibition of growth (Sharma et al. 1985). It is thought that the mechanism whereby ethylene reduces aflatoxin biosynthesis could be a receptor/sensor-mediated signaling

pathway since 1-MCP, which has a structure similar to ethylene, blocks the ethylene receptors of higher plants, and promotes aflatoxin production (Sharma et al. 1985, Serek et al. 1995, Roze 2004). Additional atmospheric ethylene prevents aflatoxin accumulation and sexual development in *A. parasiticus* and *A. nidulans*, respectively (Roze 2004). This reduction is further enhanced in *A. parasiticus* by the addition of CO_2 (Roze 2004). It is likely that the fungi may have different responses to ethylene due to adaptive survival mechanisms within the two *Aspergillus* species (Roze 2004).

Table 4. Summaries of Apple Controlled Storage Studies and Development of Patulin

Type of Study	Patulin Level	Type of Fruit or Medium	Apple Variety	Duration	References
Controlled-atmosphere-stored apples: 0-2°C					
Tree-picked	ND	Apples	MacIntosh, Red Delicious, Golden Delicious, Gala, Fuji, Red Rome, Granny Smith		Jackson et al. 2003
Dropped/Ground Harvested	40.2-374	Apples	MacIntosh, Red Delicious, Golden Delicious, Gala, Fuji, Red Rome, Granny Smith		Jackson et al. 2003
Culled	0-15.1 ug/L	Apples/*P. expansum*	MacIntosh, Red Delicious, Golden Delicious, Gala, Fuji, Red Rome, Granny Smith	4-6 Weeks	Jackson et al. 2003

*ND = Not Detected

Table 4. Summaries of Apple Controlled Storage Studies and Development of Patulin (Continued)

Unculled	59.9-120.5 ug/L	Apples/*P. expansum*	MacIntosh, Red Delicious, Golden Delicious, Gala, Fuji, Red Rome, Granny Smith	4-6 Weeks	Jackson et al. 2003
1 Month Storage of apples at		*P. expansum*:			
7°C	933-10,000 ug/l	Culture Medium			Taniwaki 1989
25°C	472-144,444 µg/l	Culture Medium			Taniwaki 1989
35°C	53.3-400 µg/l	Culture Medium			Taniwaki 1989
4°C	100-300 µg/l	Apples			Taniwaki 1989
25°C	150-311 µg/l	Apples			Taniwaki 1989
2-3 days after 5 months of cold storage (4°C)	1g/kg	Apples and Pears			Frank HK 1977
4 Months at 1°C		Pears/*P. expansum*			Frank 1977
12 Days on mineral salt solution with sugar:					
4°C	40 ug/ml	Pears/*P. expansum*			Frank 1977
25°C	3 ug/ml	Pears/*P. expansum*			Frank 1977

*ND= Not Detected

Table 4. Summaries of Apple Controlled Storage Studies and Development of Patulin (Continued)

1%CO_2, 3%O_2, and 96% N, 33°F:					
P. expansum NRRL 973	ND	Apples	Red Delicious, Golden Delicious, McIntosh	3-6 Months (Inoculated)	Lovett et al. 1975
P. expansum 1071	Detected	Apples	Red Delicious, Golden Delicious, McIntosh	3-6 Months (Inoculated)	Lovett et al. 1975
P. expansum NRRL 2034:					
0°C	ND	Pear and Apple Cultivars			Paster 1995
3°C	Detected	Pear and Apple Cultivars			Paster 1995
6°C	Detected	Pear and Apple Cultivars			Paster 1995
17°C	Detected	Pear and Apple Cultivars			Paster 1995
25°C	ND	Pear and Apple Cultivars			Paster 1995
3%CO_2/2%O_2 (25°C)	ND	Pear and Apple Cultivars			Paster 1995
3% CO_2/10% O_2	ND	Pear and Apple Cultivars			Paster 1995
3% CO_2/20% O_2	ND	Pear and Apple Cultivars			Paster 1995

*ND = Not Detected

Table 4. Summaries of Apple Controlled Storage Studies and Development of Patulin (Continued)

P. expansum NRRL 6069:					
0°C	Detected	Pear and Apple Cultivars			Paster 1995
3°C	Detected	Pear and Apple Cultivars			Paster 1995
6°C	Detected	Pear and Apple Cultivars			Paster 1995
17°C	Detected	Pear and Apple Cultivars			Paster 1995
25°C	Detected	Pear and Apple Cultivars			Paster 1995
3%CO_2/2%O_2 (25°C)	ND	Pear and Apple Cultivars			Paster 1995
3% CO_2/10% O_2 (25°C)	Detected	Pear and Apple Cultivars			Paster 1995
3% CO_2/20% O_2 (25°C)	Detected	Pear and Apple Cultivars			Paster 1995
P. expansum CBS 481.84:					
0°C	Detected	Pear and Apple Cultivars			Paster 1995
3°C	Detected	Pear and Apple Cultivars			Paster 1995
6°C	Detected	Pear and Apple Cultivars			Paster 1995
17°C	Detected	Pear and Apple Cultivars			Paster 1995
25°C	Detected	Pear and Apple Cultivars			Paster 1995
3%CO_2/2%O_2 (25°C)	ND	Pear and Apple Cultivars			Paster 1995

*ND= Not Detected

Table 4. Summaries of Apple Controlled Storage Studies and Development of Patulin (Continued)

3% CO_2/10% O_2 (25°C)	Detected	Pear and Apple Cultivars			Paster 1995
3% CO_2/20% O_2 (25°C)	Detected	Pear and Apple Cultivars			Paster 1995

*ND = Not Detected

1.9 Methods to Reduce Patulin Concentrations

Table 5 outlines various studies which have examined methods to eliminate or reduce the concentration of patulin. This section will briefly review studies examining the effects of apple trimming and culling, the use of chemical additives, fermentation, clarification and filtration, thermal processing, and storage time as means to control patulin concentrations in apple products.

1.9.1 Culling and Trimming

Trimming and culling of incoming fruit is the most effective method to reduce patulin in the resulting juice (Lovett et al. 1975). Trimming and culling has been shown to remove 93-99% of total patulin in fungus-rotted apples (Lovett et al. 1975; FDA 2001). However, culling and trimming of fruit may not be a completely reliable means to control patulin concentrations, as a lack of visible rot does not necessarily indicate the absence of patulin in the fruit (Taniwaki 1992). Adequate culling of apples prior to juice preparation takes a lot of diligence and time and is often disregarded or poorly executed. Although over-ripened or rotting fruit can be removed, sorting based on visual observation is sometimes inadequate, resulting in patulin-contaminated finished products (Harwig 1978). Washing reduces patulin concentrations in apples by 10-99%, depending on initial patulin levels and the type of wash solution used (Acar et al. 1998, Jackson 2003).

1.9.2 Chemical Additives

The inhibition of *P. expansum* by chemical additives has been studied (Ryu and Holt 1993). Patulin production was impeded when punctured apples were dipped into

solutions containing either 0.3% cinnamon oil or 0.5% potassium sorbate after inoculation with *P. expansum* (Ryu and Holt 1993). Patulin production was impeded when punctured apples were dipped into solution of 0.3% cinnamon oil or 0.5% potassium sorbate after inoculation with *P. expansum* (Ryu and Holt 1993). The authors recommended that fresh juice and cider products be surface treated with cinnamon oil or potassium sorbate like postharvest apples (Ryu and Holt 1993).

Patulin may be destroyed when sulfur dioxide is used as an antioxidant or antimicrobial agent because of its strong oxidizing capacity (Pohland and Allen 1970). SO_2 and patulin interact by the opening of the hemiacetal ring of patulin and binding of SO_2 to its double bond. However, the practical efficacy of SO_2 to reduce patulin concentrations in juice has been questioned, as Burroughs (1977) noted that the affinity of patulin to SO_2 is of little significance at the SO_2 concentrations (less than 200 ppm) normally utilized in apple juice and cider processing (Burroughs 1977). Patulin is virtually eliminated by treatment with 2,000 ppm SO_2 (Burroughs 1977).

The biological activity of patulin is diminished by binding with molecules containing a sulfhydryl group (Lindroth 1980, Martins 2002). Adducts formed when patulin reacts with sulfhydryl compounds, such as cysteine, glutathione and proteins, have diminished toxicity compared to the parent toxin. The bacteriocidal effect of patulin was partially inactivated after its reaction with SH compounds (Geiger and Conn 1945, Rinderknecht et al. 1947). Administration of patulin-glutathione at a concentration equal to the LD_{50} for free patulin had little or no toxic effect to mice, chicken embryos, and rabbit skin (Hofmann et al. 1971). Patulin-cysteine mixtures were not toxic when

injected intraperitoneally in mice at levels corresponding to 4 times the LD_{50} or when injected into chicken embryos at 50 times the LD_{50} (Ciegler et al. 1976a).

Adding ascorbate was observed to increase the rate of disappearance of patulin from apple juice and buffer solution (Brackett and Marth 1980). The mechanism of this disappearance is unknown (Lindroth 1980). However, it is assumed that a metal-catalyzed oxidation of ascorbate would somehow change the structure of patulin and account for a decrease in the detection of patulin. A clarification process is recommended before apple juice is treated with ascorbate and/or ascorbic acid to reduce patulin levels to reduce the possible regeneration of the toxin, and the possible toxicity of the reaction products. Sensory tests should be conducted to determine the acceptability of this further processing by consumers.

1.9.3 Fermentation and Clarification

Patulin is practically undetected in the presence of *Saccharomyces cerevisiae* after 3 - 14 days of fermentation (Harwig et al. 1973, Burroughs 1977). However, it should be noted that patulin has been found in some fermented products (Leggott and Shephard 2001). While clarification procedures, as well as pressing followed by centrifugation have also been found to significantly decrease the presence of patulin in juice, this increases the patulin content of pulp which may be used in animal feed (Martins 2002, Bissessur et al. 2001). Activated charcoal has been used to effectively to remove patulin from apple cider (Sands et al. 1976). It should be noted that drastic color losses have been observed with the use of some of these clarification methods (Sands et al. 1976).

One study showed that patulin is undetectable from apple juice fermented by *Saccharomyces spp* (Harwig et al. 1973). This patulin reduction by fermentation was

confirmed in studies by Burroughs (1977) and Stinson et al. (1979). This converted toxin was studied with ^{14}C-patulin (Stinson et al. 1979). The products were mostly nonvolatile and water soluble. Little, if any, patulin was shown to be metabolized to CO_2, and at least 58% of the patulin was converted to substances other than adducts of cysteine, peptides, and proteins (Lindroth 1980).

1.9.4 Biocontrol and Irradiation

The use of biocontrol and irradiation to control patulin was also studied. Castoria et al. (2005) found that *Rhodotorula glutinis* strain LS11 decreased the recovery of patulin when plated together in 96-well microtiter plates and co-inoculated into apples for 10 days. Patulin production from irradiated (100-200 Krads) spores was significantly lower than that from untreated spores (Bullerman and Hartung 1975). When mycelia were irradiated, patulin production was even lower than that from irradiated spores (Bullerman and Hartung 1975). Patulin production was not completely inhibited at either level of irradiation (Bullerman and Hartung 1975).

1.9.5 Thermal Processing

Patulin is relatively stable to heat treatment, although some decrease is noted. One study found that 50-60% of patulin was destroyed when canned apple juice was heated at 80°C for 20 minutes (Scott and Somers 1968). Another study reported when that apple juice was pasteurized at 87-89°C, the patulin content was reduced by 20% (Stray and Nossen 1978). In another study, patulin concentration in apple juice and sauce was unchanged after 30 minutes at 100°C (Andersson and Josefsson 1979). Thirty minutes of boiling did destroy 23-48% of patulin in blackcurrants, blueberries, and strawberries, and

10-20% of patulin in corresponding berry jams (Andersson and Josefsson 1979). Thermal destruction (105-125 °C) of patulin was nil in a citric acid-phosphate buffer with pH values ranging from 3.5-5.5 (Lovett and Peeler 1973).

Table 5. Summaries of Studies and on Methods to Reduce or Eliminate Patulin

Type of Intervention	Level of Reduction	Type of Product	References
Trimming/Culling	93-99%	Apples	Lovett et al. 1975
Activated Charcoal	Highly Efficient	Apple Cider	Sands et al. 1976
Ascorbate and Ascorbic Acid	Highly Efficient	Apple Juice	Brackett and Marth 1980
Irradiation 100 Krad 200 Krad 100 Krad 200 Krad	 Complete Complete 0.2 μg/mg Detected Complete	 Penicillium patulum NRRL 989 Penicillium patulum M108	 (Bullerman and Hartung 1975) (Bullerman and Hartung 1975)
Ultrafiltration	25%	Apple Juice	Acar 1998
Clarification and Filtration	39%	Apple Juice	Acar 1998
Fermentation (Yeast, Saccharomyces spp.)	Complete	Apple Juice	Burroughs 1977, 57, 142
Sulfhydryl Compounds (Adducts formed with cysteine, glutathione, etc.)	4-100 times less toxic	Chicken Embryo	Ciegler et al. 1976, Lindroth and von Wright 1978, von Wright and Lindroth 1978
Sulfur Dioxide	Highly Efficient	Apple Juice and Cider	Pohland and Allen 1970, Burroughs 1977
3% Cinnamon Oil	Highly Efficient	Apples	Ryu and Holt 1993
0.5% Potassium Sorbate	Highly Efficient	Apples	Ryu and Holt 1993
Cooking/Temperature: 10 min 30 min Boiling Boiling	 90% No change 23-48% 10-20%	 Apple Puree Apple Juice and Sauce Blackcurrants, Blueberries, Strawberries Corresponding Berry Jams	 Frank et al. 1976 Andersson and Josefsson 1979 Lindroth 1980ab Lindroth 1980ab
Canning (80°C for 20 min)	50-60%	Apple Juice	Scott and Somers 1968
Pasteurization (87-89°C)	20%	Apple Juice	Stray and Nossen 1978
High Pressure Washing	54%	Apples for Apple Juice	Acar et al. 1998

1.10 Patulin Regulations

Patulin regulations were needed because small amounts of *P. expansum* contamination of apples can lead to high patulin concentrations in processed apple products (McCallum et al. 2002). Regulation was especially important because of the high prevalence of patulin-contaminated products in commerce (Essa and Ayesh 2000). The World Health Organization (1995) has determined the tolerable daily human intake of patulin should not exceed 50 μg/kg body weight/day, using a 100-fold safety factor. The current Codex Alimentarius Commission (2003) recommended maximum residue limits (MRLs) for patulin is 50 μg/kg in apple juice and in beverages containing apple juice ingredients.

Several countries worldwide have regulations for the control of patulin in fruit juices and some fruit products (Majerus 2002). Table 6 outlines individual countries' patulin MRLs for specific products. Although most of the regulations only pertain to patulin concentrations in clear juices, the need for patulin control in cloudy juices and apple puree has been sought by the European Union since damaged and moldy apples may be used in the production of these products (MacDonald et al. 2000).

The U.S. FDA also adopted the 50μg/L MRL for patulin in apple juice, apple juice concentrate and apple sauce (FDA 2001). This was based upon "no observed adverse effect level" (NOAEL), provisional tolerable daily intake (PTDI), and provisional tolerable weekly intake (PTWI) estimates developed based on animal toxicity studies (Becci et al. 1981, FDA 2000). The PTDI for patulin was calculated to be 0.43 μg/kg body weight based upon 90th percentile patulin exposure for all ages (FDA 2000).

1.10.1 Juice HACCP

There are an estimated 16,000 to 48,000 juice-associated illnesses per year in the United States (FDA 2001). Most of these juice-associated illnesses are suspected to be caused by pathogenic microorganisms, especially in unpasteurized fruit juices (Besser et al. 1993, FDA 2001). These facts and several recalls led the FDA to address foodborne illness and hazards related to juice products (FDA 1997). A warning statement on juice product labels was required in 1998 if they were not processed using Hazard Analysis Critical Control Point (HACCP) or a lethality step that would lead to a 5-log reduction of the pertinent pathogens (FDA 1998). Furthermore, in 1998 FDA stated an intent to require juice processors to develop and implement HACCP systems (FDA 1998). To heighten juice manufacturers' knowledge, educational programs on juice safety and HACCP were established (Thede 2004).

The FDA required HACCP procedures to be implemented by all the juice processors by 2004 (FDA 2001), but the effects of FDA HACCP requirements on patulin contamination in juice products have not been reported. In 2003, two recalls of 1,500 and 38,000 bottles of apple juice were issued by FDA for noncompliant patulin levels (ODH 2003). This shows further need for patulin surveillance and improved patulin controls in apple juice products.

Table 6*. Present Regulations for Patulin in EU Member States and the United States

Country	Commodities	Maximum limit (µg/kg)
Austria	Fruit juice	50
Finland	All foods	50
France	Apple juice and derived products	50
Germany	Apple juice	50
Italy	Fruit juice	50
Norway	General	50
Sweden	Berries and products of berries	50
United Kingdom	Apple juice	50
United States	Apple juice, Apple juice concentrate and Apple sauce	50

*From Majerus et al. 2002

1.11 Objectives and Specific Aims

Based upon this review of the literature, objectives and specific aims were developed. The **overall objectives** of this research were to determine the prevalence and levels of patulin contamination in apple cider and juice in Michigan and to assess factors influencing patulin production in apples and its resulting concentration in apple juice products. In order to meet these overall objectives, experiments were conducted to achieve the following **specific aims**:

1. Determine the concentrations of patulin in a) apple cider produced and marketed by Michigan cider mills in 2002, 2003, and 2004 and b) different brands of apple juice and cider, including shelf-stable products, marketed in retail grocery stores in Michigan in 2005 and 2006.
2. Determine the production of ethylene by *Penicillium expansum* and the relationship between the presence of endogenous and exogenous ethylene and patulin production by *Penicillium expansum* on liquid and solid media.

3. Determine whether fruit-produced ethylene or exogenous ethylene (100 μL/L) affects the production of patulin in apples treated with 1-methylcyclopropene (1-MCP) and inoculated with *Penicillium expansum*.
4. Determine the effect of trimming and culling of apples inoculated with *Penicillium expansum* on patulin levels in intact and decayed apple tissue and in juice produced from two varieties of apples.

The overall outcome of this study will be to increase the awareness of the apple industry, allowing them to take preventative action, which will lead to cost savings and a decrease in potential recalls and adverse publicity.

1.12 References

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2 Patulin Surveillance in apple cider produced and sold by Michigan cider mills in 2002-2004 and apple juice and cider sold in retail grocery stores in Michigan in 2005-2006

2.1 Abstract

Patulin is the most common mycotoxin found in apples and apple juices. Because of its toxicity, the FDA has established that the patulin concentration of apple juice products should not exceed 50 μg/L. The objective of this study was to determine the concentrations of patulin in a) apple cider produced and marketed by Michigan apple cider mills in 2002, 2003, and 2004 and b) apple juice and cider, including shelf-stable products, marketed in retail grocery stores in Michigan in 2005 and 2006. End-product samples (N = 493) of 104 Michigan apple cider mills were obtained in 2002, 2003, and 2004 and analyzed for patulin concentration using solid phase extraction followed by high performance liquid chromatography. The majority (81.3%) of apple cider produced by Michigan mills in 2002-2004 contained no detectable patulin. Patulin was detected (≥4 μg/L) in 18.7% of all cider mill samples with 11 (2.2%) samples having patulin concentrations above the legal limit of 50 μg/L. A greater percentage of cider samples obtained from mills using thermal pasteurization contained detectable patulin (28.4%) when compared to mills using ultraviolet-light irradiation (13.5%; $P \leq 0.006$) or no pathogen reduction treatment (17.0%; $P \leq 0.02$). Among juice and cider samples obtained from retail grocery stores (N=159), 23% contained detectable patulin, with 18 (11.3%) samples having patulin concentrations above the legal limit of 50 μg/L. Some apple juice samples obtained from retail grocery stores had exceptionally high patulin concentrations, ranging up to 2700 μg/L. Collectively, these results indicate that some members of apple cider and juice industries have inadequate controls over patulin

concentrations in products. The industry, overall, should focus on improved quality of fruit used in juice production and improve culling procedures to reduce patulin concentrations.

2.2 Introduction

Patulin, a secondary fungal metabolite produced by several species of *Penicillium*, *Aspergillus*, and *Byssochlamys*, is the most common mycotoxin found in apples and apple juices (Wilson 1974, Lindroth 1980, Martins 2002). *Penicillium expansum*, which is commonly found in soil, is the primary source of patulin in fruit (Askar 1999, Jackson et al. 2003). Patulin causes gastrointestinal toxicity in rodents and also has mutagenic, neurotoxic, genotoxic and carcinogenic effects in rodents (Dickens and Jones 1961, Osswald 1978, Ciegler 1976, Reddy 1978, Reddy 1979, Lindroth 1980). Furthermore, patulin causes dermal and gastric irritation in humans and also suppresses the immune system of rodents, producing ulceration, congestion, and hemorrhagic lesions, especially in the gastrointestinal tract (Dickens and Jones 1961, McKinley et al. 1980ab, DeRosnay 1952, Dalton 1952). Based upon its toxicity profile, an action level of 50 μg/kg was established for patulin in apple juice, apple juice concentrate (at single strength dilution), and apple sauce (FDA 2001).

Patulin surveillance in apples and apple juices by regulatory authorities in the U.S. and other countries has resulted in a significant number of product recalls due to patulin concentrations in excess of regulatory limits. In fact, several recent recalls of juice products due to excessive patulin concentrations have occurred in the U.S. and U.K. Thus, patulin contamination of food products has significant economic implications for food processors in addition to its detrimental effects on public health. Establishment and enforcement of maximum residue levels (MRLs) for patulin in apple juice and cider in other countries has resulted in its reduced prevalence in products. For example, a 1998 United Kingdom study reported that the percentage of U.K. apple juice samples that

contained patulin concentrations greater than 50 μg/L decreased from 26% to 2% over a six-year period following the establishment of the action level in the UK (MAFF 1998).

Previous studies have surveyed patulin concentrations in apple cider and juices in the U.S. For example, the U.S. Food and Drug Administration surveyed patulin in apple juice in the U.S. market in 1973, and patulin was detected in 37% of 136 samples with an average contamination level of 69 μg/L (40-440 μg/L range) (Stoloff 1976). In Washington, D.C. area stores, 8 out of 13 apple juices contained patulin with concentrations of 44-309 μg/L (Ware et al. 1975). On roadside stands in Wisconsin, patulin was detected in 29 out of 66 samples of apple juice (Brackett and Marth 1980).

Statewide surveillance of patulin concentrations in apple cider produced by Michigan cider mills has not been previously conducted. Furthermore, recent data on patulin concentrations in U.S. apple juice products are not available. We were particularly interested in assessing patulin contamination of apple cider and juice products in the years during and immediately subsequent to implementation of FDA's juice HACCP regulation (1998, FDA 2001) and establishment of the U.S. action level for patulin in apple juice, apple juice concentrate, and apple sauce (FDA 1998; FDA 2001). Therefore, the objectives of this research were to determine the concentrations of patulin in apple juice and cider a) produced and marketed by apple cider mills in Michigan in 2002, 2003, and 2004 and b) in different brands of apple juice and cider, including shelf-stable products, marketed in retail grocery stores in Michigan in 2005 and 2006.

2.3 Materials and Methods

Samples of Michigan apple cider were purchased at 59, 104, and 74 cider mills and retail establishments throughout the state of Michigan in 2002, 2003, and 2004,

respectively. All samples (0.473 – 3.875 L) were obtained within 14 days of manufacture during cider season and prior to any "sell by" or "use by" dates on product packages.

The samples were stored on ice or refrigerated and immediately delivered to the Michigan Department of Agriculture (MDA) Region Six Laboratory in East Lansing, MI for microbial analyses. Results of these microbial analyses are published elsewhere (Bobe et al. 2007). The MDA saved aliquots (at least 50 ml) of the samples, which were frozen and returned to MSU for patulin testing in our laboratory.

We also obtained samples of apple juice and cider (including shelf-stable products) produced by large juice processing facilities (including many national brands) and sold in retail grocery stores in Michigan at four times during 2005 and 2006. Apple juice and cider products (31, 24, 37, and 32 samples obtained in Summer 2005, Fall 2005, Spring 2006, and Summer 2006, respectively) were purchased and aliquots were placed into 50 ml centrifuge tubes and frozen until patulin analysis. To determine patulin concentrations, all samples were subjected to solid phase extraction and patulin was quantified by high performance liquid chromatography. Samples were analyzed in triplicate and analyses were repeated if the standard deviation of the three samples was more than 10% of the mean.

2.3.1 Solid-Phase Extraction

Solid-phase extraction (SPE) of patulin from apple juice was carried out using a syringe-cartridge method (Eisele and Gibson 2003). The SPE cartridge (Waters Oasis® HLB 60 mg, Milford, MA) was activated by applying 2-3 ml of water, followed by 2 ml of methanol and a final wash of 2-3 ml of water. Each sample (2.5 mL volume) was then applied to cartridges under vacuum. The cartridges were rinsed with 2.0 ml of 1.0%

sodium bicarbonate solution (pH=8.3) followed by 2 ml of 1% acetic acid solution. One milliliter of 10% ethyl acetate in ethyl ether solution was then added to each cartridge to elute patulin. The eluates were collected in 15x45 mm vials, evaporated to dryness under a stream of nitrogen and then reconstituted in 0.5 ml of 0.1% acetic acid. The vials were capped, vigorously mixed, and frozen for future analysis by HPLC.

2.3.2 High Performance Liquid Chromatography (HPLC)

Patulin was quantified using a Waters Corporation (Milford, MA) high performance liquid chromatograph (HPLC). The Waters system included two model 510 pumps, a model 717-plus auto-sampler, and a model 996 Photodiode Array detector. An Alltech C_{18}, 5 μm column (4.6 x 250 mm) was used to separate compounds. Each sample (50 μL injection volume) was subjected to isocratic elution for 30 minutes with 10% aqueous acetonitrile (ACN), followed by a 30-minute clean with 70% of 100% ACN and 30% of 10% ACN. The photodiode array detector collected the UV and visible light spectrum from 190 to 800 nm to allow comparison of sample UV spectra to that of patulin standards. Chromatograms were analyzed using Waters' Millenium software. Patulin concentration was quantified by comparing UV absorbance at 276 nm to known patulin standards.

2.3.3 Statistical Analyses

For statistical analysis, SAS Version 9.1 was used (SAS Institute Inc., Cary, NC). The proportion of samples containing detectable patulin and the proportion of samples containing patulin concentrations above the legal limits were compiled for cider mills who used thermal processing, UV-light treatment, or no pathogen reduction technology.

These same data also were stratified based on year of production (2002, 2003, 2004). In addition, the relationship between the presence of microorganisms and pathogen reduction measures (none, thermal pasteurization, UV light irradiation) and patulin concentration was examined in the cider mill samples obtained in 2002-2004. The average proportion of samples that tested positive for patulin was compared among the groups by using chi-square tests in PROC GENMOD in SAS. The same procedure was used to compare the average proportion of samples that exceeded the legal limit of 50 μg/L to counts for generic *E. coli*, total coliforms, or aerobic microorganisms. We evaluated the data by comparing the average $\log_{10}$-transformed counts of samples that tested positive for *E. coli* O157:H7, total coliforms, or aerobic microorganisms, as well as the patulin concentration, respectively, between the groups using two-sided t-tests in PROC MIXED in SAS. The same statistical procedures were used to evaluate the impact of year of processing (2002, 2003, and 2004) on microbiological criteria in Michigan apple cider. In Table 7, means ± standard error of means (SEM) of proportions of samples testing positive and $\log_{10}$-transformed counts are presented and significant differences between groups at $P \leq 0.05$ and tendency for differences between groups at $P \leq 0.10$ are designated by different superscripts, a,b,c and d,e,f, respectively. The same statistical procedures were used for analyzing patulin concentrations in samples obtained from the retail grocery stores, but this information was further explored based on ingredients used, beverage type (juice vs. cider), handling (refrigerated vs. shelf stable), production system (organic vs. conventional), and time of sampling.

2.4 Results

2.4.1 Cider Mill Samples

Results of patulin surveillance conducted on juice and cider samples obtained from Michigan cider mills are presented in Table 7. The majority of apple cider and juice produced in Michigan in 2002-2004 contained no detectable patulin. When all 2002-2004 Michigan cider mill patulin surveillance data are combined (N=493), patulin was detected (≥4 μg/L) in 18.7% of all cider samples with 11 (2.2%) samples having patulin concentrations above the legal limit of 50 μg/L. The average concentration of patulin in samples that contained detectable patulin was 36.9 μg/L, with a range of 4.6 to 467.4 μg/L.

We also examined the effect of pathogen control measures and year of processing on patulin concentrations (Table 7). Among samples in which no pathogen control measure was used (n=324), 17.0% of all samples had detectable patulin and 2.8% (9 samples) were above the legal limit. The average patulin concentration of the samples containing detectable patulin was 42.1 μg/L with a range of 4.8 to 329.8 μg/L. Among samples that were thermally pasteurized (n=95), 28.4% contained detectable patulin with 1.1% (1 sample) above the legal limit. The average concentration of the samples containing detectable patulin was 33.4 μg/L with a range of 4.6 to 467.4 μg/L. Among samples treated with UV-light irradiation, 13.5% contained detectable patulin with 1.4% (1 sample) above the legal limit. The average concentration of samples containing detectable patulin was 17.3 μg/L, with a range of 5.5 to 59.1 μg/L. A significantly greater percentage of samples obtained from facilities using thermal pasteurization contained detectable patulin (28.4%) compared to facilities using ultraviolet-light

irradiation (13.5%; $P \leq 0.02$) or no pathogen reduction technology (17.0%; $P \leq 0.01$). However, there was no difference among these treatments in likelihood of samples containing patulin concentrations ≥50 μg/L.

Bacterial counts detected in these apple cider samples and the influence of processing procedures (e.g. thermal processing, ultraviolet light processing) used by cider mills on bacterial counts were reported by Thede et al. (2004) and Bobe et al. (in press). Detectable bacterial counts (total aerobic plate count, total coliforms, and generic *E. coli*) were not associated ($P \geq 0.10$) with $\log_{10}$ transformed patulin concentrations in these samples (data not shown).

The influence of cider production year on patulin concentrations is reported in Table 7. Among samples obtained in 2002 (n=71), 21.1% contained detectable patulin, with 4.2% (3 samples) above the legal limit. The average of patulin concentrations and range were 55.2 μg/L and 4.9 to 329.8 μg/L, respectively. Among the 2003 samples (n= 241), 12.9% contained detectable patulin, with 0.8% (2 samples) above the legal limit. The average and range of patulin concentrations were 23.9 μg/L and 4.8 to 96.4 μg/L, respectively. Among 2004 samples (n= 181), 25.4% contained detectable patulin, with 3.3% (5 samples) above the legal limit. The average of patulin concentrations and range were 39.7 μg/L and 4.6 to 467.4 μg/L, respectively.

Apple cider samples obtained in 2003 were significantly ($P \leq 0.05$) less likely to contain patulin than those obtained in 2004, and tended ($P \leq 0.10$) to be less likely to contain patulin when compared to 2002 samples. There was a tendency ($P \leq 0.10$) for more samples obtained in 2002 and 2004 to contain ≥ 50 μg/L patulin when compared to samples obtained in 2003.

Table 7. Patulin Concentrations (Log_{10} µg/L) in Michigan Apple Cider Samples, 2002-2004 – Overall and Stratified by Pathogen Reduction Measures and Year of Processing

Food safety control measures/ Year of processing	No. of samples	Samples containing detectable patulin (%)[1]	Samples containing patulin ≥50 µg/L (%)[1]	Average concentrations of samples with detectable patulin (µg/L)[1]	Range of concentration of samples with detectable patulin (µg/L)[1]
Total	493	18.7 ± 1.8	2.2 ± 0.7	36.9 ± 7.2	4.6 – 467.4
Food safety control measures					
Untreated	324	17.0^{b} ± 2.1	2.8 ± 0.9	42.1 ± 8.7	4.8 – 329.8
Thermal Pasteurization	95	28.4^{a} ± 4.7	1.1 ± 1.1	33.4 ± 16.8	4.6 – 467.4
UV light irradiation	74	13.5^{b} ± 4.0	1.4 ± 1.4	17.3 ± 6.1	5.5 – 59.1
Year of processing					
2002	71	21.1^{ab} ± 4.9	4.2^{d} ± 2.4	55.2 ± 23.9	4.9 – 329.8
2003	241	12.9^{b} ± 2.2	0.8^{e} ± 0.6	23.9 ± 3.6	4.8 – 96.4
2004	181	25.4^{a} ± 3.2	3.3^{d} ± 1.3	39.7 ± 11.9	4.6 – 467.4

[1] Least square means ± standard errors are shown.

[a,b,c] Numbers with different superscripts (a,b,c) within a column and within pathogen reduction measures or year of processing significantly differ at $P \leq 0.05$.

[d,e] Numbers with different superscripts (d,e) within a column and within pathogen reduction measures or year of processing tend to differ at $P \leq 0.10$.

2.4.2 Retail Samples

Data on patulin concentrations in apple juice samples obtained from Michigan retail grocery stores are summarized in Tables 8 and 9. The majority of apple juice and apple cider samples (N=159) obtained from Michigan retail establishments in 2005 and 2006 had non-detectable patulin concentrations (Table 8). However, 23.3% of all cider samples contained detectable (≥4 μg/L) patulin, with 18 (11.3%) samples having patulin concentrations above the legal limit of 50 μg/L. The average patulin concentration (± standard error) of patulin-positive retail samples was 226 ± 85 μg/L, respectively. The range of patulin concentrations in positive samples was 8.8 to 2700 μg/L.

These samples were also categorized and statistically analyzed to compare ingredients used (apples, concentrate, or both), beverage type (juice or cider), production system (organic vs. conventional), handling (shelf-stable or refrigerated), time of sampling (Summer 2005, Fall 2005, Spring 2006, or Summer 2006), and whether apples were used for juice processing in-season or off-season. Among juice samples manufactured from apples (n=49), 40.8% contained detectable patulin, 9 (18.4%) of which were above the legal limit. Among juice samples made from concentrate (n=99), 13.4% contained detectable patulin, 7 (7.1%) of which were above the legal limit. According to the statement of ingredients on the product labels, 11 samples used both apples and concentrate as ingredients. Among these samples, 36.4% contained detectable patulin, 2 (18.2%) of which were above the legal limit. Juices made from apples were more likely to contain detectable patulin ($P = 0.0003$) than juices made from concentrate. Juices made using both apples and concentrate tended to be more likely to contain detectable patulin ($P = 0.0553$) than samples made from concentrate. Similarly, juices

made from apples were more likely (P = 0.0440) to have patulin levels above 50 μg/L when compared to juices manufactured from concentrate.

Among the cider samples that had not been clarified (n=18), 27.8% contained detectable patulin, with none above the legal limit of 50 μg/L (Table 8). Among the juice samples (n=141), 22.7% contained detectable patulin, with 12.8% (18 samples) above the legal limit. Compared to products designated as ciders (all of which were manufactured using apples as an ingredient), juice products were significantly more likely to contain patulin concentrations exceeding the legal limit. The average concentration of patulin in juice samples also tended (P < 0.10) to be greater than that observed in ciders.

Of the juices labeled as organic (n=14), 42.9% had detectable patulin with 21.4% (3 samples) above the legal limit. Of the samples produced using conventionally grown ingredients (n=145), 21.4% had detectable patulin with 10.3% above the legal limit. There was a tendency (P=0.08) for organic juices to be more likely to contain detectable patulin when compared to conventional juices.

Among refrigerated retail juices (n=29), 24.1% contained detectable patulin, 1 (3.4%) of which was above the legal limit (Table 8). Among shelf-stable retail juices (n=130), 23.1% contained detectable patulin, 17 (13.1%) of which were above the legal limit. Shelf-stable samples showed a tendency (P = 0.0711) to be more likely to contain illegal levels of patulin when compared to refrigerated samples.

Comparison of patulin concentrations of juices obtained at retail outlets at different times are summarized in Table 9. No differences were detected in the proportion of samples containing detectable patulin, the proportion of samples containing > 50 μg/L patulin, or the mean patulin concentration when comparing the four sampling

periods. Similarly, no differences in these parameters were detected when samples were categorized as being obtained during apple harvest season (Fall 2005 juices only) or in the off-season (combined results for Summer 2005, Spring 2006, and Summer 2005 sampling periods). However, off-season-procured juices made from apples were significantly more likely to contain detectable patulin ($P \leq 0.02$) when compared to juices produced from apples during apple harvest season. When broken down by ingredients and time, of those juices produced from apples during the harvest season, 9.1% (1 sample) contained detectable patulin. This juice sample also happened to exceed the legal limit. Of those juices produced from apples during the off-season, 50.0% contained detectable patulin, 8 (21.1%) of which were above the legal limit. Among those juice samples produced from concentrate, obtaining samples during apple harvest season or in the off-season did not influence patulin detection frequency, the proportion exceeding the legal limit, nor the mean patulin concentration.

Table 8. Patulin Concentrations in Retail Samples Sold in the State of Michigan 2005-2006 – Overall and Stratified by Beverage Type, Growth Treatment, Handling, Ingredients

Ingredients/ Beverage Type/Production System/Handling in Distribution	No. of samples	Samples containing detectable patulin (%)[1]	Samples containing patulin ≥50 µg/L (%)[1]	Average concentrations of samples with detectable patulin (µg/L)[1]	Range of concentration of samples with detectable patulin (µg/L)[1]
Total	159	23.3 ± 3.4	11.3 ± 2.5	226.0 ± 85.4	8.8 – 2700.4
Ingredients:					
Apples	49	40.8[a] ± 7.1	18.4[a] ± 5.6	85.2[d] ± 25.5	8.8 – 417.6
Both	11	36.4[ab] ± 5.2	18.2[ab] ±12.2	128.4[de] ± 78.8	13.6 – 347.4
Concentrate	99	13.4[b] ± 3.7	7.1[b] ± 2.6	472.7[e] ± 228.7	13.4 – 2700.4
Beverage Types:					
Cider	18	27.8 ± 10.9	<4[b]	24.2[d] ± 3.9	15.3 – 35.2
Juice	141	22.7 ± 3.5	12.8[a] ± 2.8	257.5[e] ± 97.7	8.8 – 2700.4
Production System:					
Organic	14	42.9[d] ±13.7	21.4 ± 1.4	93.9 ± 39.1	17.9 – 264.8
Conventional	145	21.4[e] ± 3.4	10.3 ± 2.5	251.6 ± 101.2	8.8 – 2700.4
Handling in Distribution:					
Refrigerated	29	24.1 ± 8.1	3.4 ± 3.4	38.0[d] ± 13.0	16.7 – 114.5
Shelf	130	23.1 ± 3.7	13.1 ± 3.0	269.9[e] ± 103.9	8.8 – 2700.4

[1] Least square means ± standard errors are shown.

[a,b,c] Numbers with different superscripts (a,b,c) within a column and within pathogen reduction measures or year of processing significantly differ at $P \leq 0.05$.

[d,e] Numbers with different superscripts (d,e) within a column and within pathogen reduction measures or year of processing tend to differ at $P \leq 0.10$.

Table 9. Patulin Concentrations in Retail Samples Sold in the State of Michigan 2005-2006 – Overall and Stratified by Time of Processing, and Season

Time of Sampling/ Season/ Ingredients	No. of samples	Samples containing detectable patulin (%)[1]	Samples containing patulin ≥50 µg/L (%)[1]	Average concentrations of samples with detectable patulin (µg/L)[1]	Range of concentration of samples with detectable patulin (µg/L)[1]
Time of Sampling:					
Summer 2005	35	28.6 ± 7.7	5.7 ± 4.0	53.8 ± 24.4	15.3 – 264.8
Fall 2005	39	23.1 ± 6.8	12.8 ± 5.4	338.1 ± 186.6	13.4 – 1722.6
Spring 2006	44	20.5 ± 6.2	13.6 ± 5.2	126.4 ± 47.5	13.6 – 417.6
Summer 2006	41	22.0 ± 6.5	12.2 ± 5.2	404.8 ± 293.3	8.8 – 2700.4
Season:					
In-season	39	23.1 ± 6.8	12.8 ± 5.4	338.1 ± 186.6	13.4 – 1722.6
Off-season	120	23.3 ± 3.9	10.8 ± 2.8	190.0 ± 96.5	8.8 – 2700.4
For apples only:					
In-season	11	9.1^{b} ± 9.1	9.1 ± 9.1	114.5	114.5
Off-season	38	50.0^{a} ± 8.2	21.1 ± 6.7	83.6 ± 26.9	8.8 – 417.6
For concentrate only:					
In-season	24	20.8 ± 8.5	8.3 ± 5.8	485.8 ± 331.9	13.4 – 1722.6
Off-season	75	10.7 ± 3.6	6.7 ± 2.9	464.5 ± 326.0	26.8 – 2700.4

[1] Least square means ± standard errors are shown.

[a,b,c] Numbers with different superscripts (a,b,c) within a column and within pathogen reduction measures or year of processing significantly differ at $P \leq 0.05$.

[d,e] Numbers with different superscripts (d,e) within a column and within pathogen reduction measures or year of processing tend to differ at $P \leq 0.10$.

2.5 Discussion

Public and governmental concern over the safe production of juice increased in 1996 in light of foodborne illness outbreaks associated with the consumption of unpasteurized apple juice and cider products. To increase juice safety and enhance

pathogen control, in 2001 FDA issued juice HACCP regulations to be implemented in all juice processing plants by January 2004. Also in 2001, FDA implemented additional regulations by establishing a maximum limit for patulin concentrations in apple juice and cider, apple juice concentrate (at single strength), and apple sauce at 50 μg/L. We were especially interested in determining the prevalence of patulin in apple juice and cider produced in mills and sold in grocery stores in the state of Michigan subsequent to FDA rulemaking on juice HACCP and patulin maximum residue requirements.

To conduct this study, we collected cider produced and sold in Michigan cider mills and juice and cider sold in retail grocery stores in Michigan, including local and national brands. To our knowledge, this is the first large-scale, multi-year surveillance study that has been conducted to estimate the frequency of patulin presence in commercial apple cider and juice samples in Michigan.

2.5.1 Cider Mill Samples

Bacterial counts in the samples obtained from cider mills and the processing procedures (e.g. thermal processing, ultraviolet light processing) used to produce these samples were reported by Thede et al. (2004) and Bobe et al. (Journal of Food Protection in press). Bacterial counts (total plate count, coliforms, and E. coli O157:H7) were not associated with $\log_{10}$ transformed patulin concentrations ($P \geq 0.10$) in these samples. However, illegal concentrations of patulin were found in 2.2% of the cider mill samples. It should be noted that samples containing patulin concentrations above 50 μg/L are legally adulterated, just as would be the case if they were found to contain pathogenic microorganisms.

A significantly greater percentage of samples obtained from facilities using thermal pasteurization contained detectable patulin (28.4%) compared to facilities using ultraviolet-light irradiation (13.5%; $P \leq 0.02$) or no pathogen reduction technology (17.0%; $P \leq 0.01$) (Table 7). This difference likely was due to the use of a greater proportion of unsound fruit by thermal processors knowing that adequate thermal processing will effectively control potential microbial hazards associated with this lower-quality fruit.

We observed significant differences in patulin contamination in the three years of sampling apple cider (Table 7). We hypothesize that differences in patulin contamination could be due to a late frost and poor apple production in 2002 and more puncture wounds in apples due to hail storms in 2004, thereby limiting fruit availability and perhaps diverting more unsound fruit into juice production in 2002 and 2004. The noticeably lower patulin incidence observed in 2003 samples might be explained by a considerably greater availability of fresh apples that year according to the Michigan Agricultural Statistics (2005-2006). Michigan apple production in 2003 (930 million pounds) was significantly greater than in 2002 (520 million pounds) and 2004 (730 million pounds). Apple processing was also higher in 2003 (580 million pounds) than in 2002 (365 million pounds) and 2004 (490 million pounds). The patulin incidence in 2002 may be slightly lower than 2004 due to fewer apples being held in cold and controlled atmosphere storage during that year (134,627,000 pounds vs. 223,445,000 pounds). Collectively, this information leads us to hypothesize that processors may compensate for lower apple production during a year by culling and trimming less, or by simply using whatever fruit is available.

2.5.2 Retail Samples

A considerably larger percentage (11.3%) of apple juice samples obtained at retail grocery stores in 2005-2006 contained patulin concentrations above the legal limit than the cider mill samples collected in 2002-2004. This observation suggests that processors supplying juice year-round to retail chains have greater problems with patulin contamination than the small cider mills. This could be due to longer storage times for fruit before juice manufacture and also due to inadequate controls of patulin concentrations in apple juice concentrates used for juice production by larger processors.

Shelf-stable samples showed a tendency to have a greater frequency of patulin contamination when compared to refrigerated samples (Table 8). This difference may be due to faster rotation of refrigerated products, and because they tend to be from more localized sources.

Juices made from apples were more likely to have detectable patulin ($P = 0.0003$) than juices made from concentrate (Table 8). Juices made using both apples and concentrate tended to be more likely to contain detectable patulin ($P = 0.0553$) than juices made from concentrate alone. Furthermore, juices made from apples were more likely ($P = 0.0440$) to have samples with patulin levels above 50 μg/L when compared to those manufactured from concentrates. However, juices manufactured using concentrates contained exceptionally high levels of patulin contamination on several occasions, indicating that some apple juice concentrate suppliers are using fruit of very low quality.

There was a tendency ($P=0.08$) for organic juices to have a greater proportion containing detectable patulin in comparison to conventionally produced juices (Table 8). Organic juices are considered more wholesome by the average consumer, but our results

(based on a relatively small number of observations) indicate that organic apple juices might be more likely to contain patulin when compared to their conventional counterparts. This is not particularly surprising since it has been found that some insecticides reduce and even inhibit patulin production (Draughon and Ayres 1979).

2.2% and 11.3% of randomly obtained apple juice samples from Michigan cider mills and retail establishments, respectively, contained >50 μg patulin/liter. This is an excessive proportion of adulterated apple juice in the marketplace two years after juice HACCP implementation. Juice processors must be encouraged to use appropriate apple storage and culling practices. It is hoped that these surveillance data will encourage processors to take further steps to reduce the likelihood of patulin contamination in juice products. We hope that increased awareness of patulin as a hazard in apples and processed apple products will enable the industry to take preventative actions which will result in decreased risk of product recalls and adverse publicity.

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3 Ethylene production by *Penicillium expansum* and its endogenous and exogenous effects on patulin production in inoculated solid and liquid media

3.1 Abstract

Patulin is produced by mold species such as *Penicillium expansum* and is the most abundant mycotoxin in apples and apple juice. Production of patulin in infected apples can be reduced by modified atmosphere fruit storage conditions. The objectives of this research were to determine the production of ethylene and patulin by *P. expansum* and the influence of exogenous ethylene and the ethylene action inhibitor, 1-MCP, on patulin production by *P. expansum* when cultured on liquid or solid media. In experiment 1, spores of *P. expansum* ATCC 28883 and/or ATCC 1117 were cultured in tubes containing PDA or PDB. *P. expansum* ATCC 28883 produced much larger concentrations of patulin and ethylene than *P. expansum* ATCC 1117. Timing of patulin and ethylene production by *P. expansum* was inversely related for both strains. The second experiment was conducted on plates containing PDA or PDB and treating *P. expansum* ATCC 28883 in a flow through system with combinations of ethylene and 1-MCP using a factorial design. Patulin production in Petri plates containing PDA inoculated with *P. expansum* ATCC 28883 were significantly increased by the presence of exogenous ethylene ($P = 0.0206$). There was no overall 1-MCP effect ($P = 0.72$) on patulin production. These results confirm that P. expansum produces ethylene and suggests an inverse relationship between endogenous ethylene and patulin production.

3.2 Introduction

Patulin, a secondary fungal metabolite produced by several species of *Penicillium*, *Aspergillus*, and *Byssochlamys* fungi, is the most common mycotoxin found in apples and apple juices (Wilson 1974, Lindroth 1980, Martins 2002). *Penicillium expansum* is the primary source of patulin in fruit (Askar 1999, Jackson et al. 2003). Patulin causes gastrointestinal toxicity in rodents and also has mutagenic, neurotoxic, genotoxic and carcinogenic effects in rodents. Furthermore, patulin causes dermal and gastric irritation in humans and also suppresses the immune system of rodents, producing ulceration, congestion, and hemorrhagic lesions, especially in the gastrointestinal tract (Dickens and Jones 1961, Moodley 2002, McKinley et al. 1980ab, Martins et al. 2002). Based upon this toxicity profile, the US Food and Drug Administration (FDA) established an action level for patulin at 50 μg/kg in apple juice and in beverages containing apple juice, apple juice concentrate (at single strength) and apple sauce (FDA 2001). In recent years, patulin surveillance in apples and apple juices by regulatory authorities in the U.S. and other countries has resulted in a significant number of product recalls due to patulin concentrations in excess of regulatory limits. Thus, patulin contamination of food products has significant economic implications for food processors in addition to its detrimental effects on public health.

It is well established that patulin is formed predominantly by *P. expansum* in apples after harvest and that patulin is commonly present in juice or cider produced from these moldy apples. Several strains of *P. expansum* have been reported to produce ethylene in non-peer-reviewed reports (Taiz and Zeiger 2002). However, the relationship between ethylene production and onset of toxin by *P. expansum* production has not been

examined. Suppression of ethylene production by *Aspergillus parasiticus* and *Aspergillus flavus* is strongly associated with the onset of aflatoxin production by these fungi (Sharma et al. 1985). Aflatoxin production by *Aspergillus parasiticus* also was found to be significantly inhibited by exposure to 100 μL/L exogenous ethylene (Roze et al. 2004). Although the biosynthesis pathways of patulin and aflatoxin by these fungi differ, they are both polyketides, and the relationship between the presence of ethylene and toxin production may be similar.

The overall objective of this research was to investigate possible associations between ethylene and patulin production by *P. expansum*. A series of experiments was conducted to answer three main questions concerning the potential relationship between ethylene and patulin: 1) do *P. expansum* strains ATCC 1117 and ATCC 28883 produce ethylene, and if so, how much? 2) when is patulin produced by *P. expansum* relative to the presence of endogenous and exogenous ethylene when grown on solid media? and 3) what is the influence of endogenous and exogenous ethylene on patulin production by *P. expansum* when cultured in liquid media?

3.3 Materials and Methods

3.3.1 Preparation of Commercial Mold Cultures

Pure cultures of *Penicillium expansum* ATCC 28883 and ATCC 1117 were obtained as freeze-dried cultures from the American Type Culture Collection (ATCC; Manassas, VA). After each capsule was aseptically opened, the freeze-dried cultures were rehydrated according to ATCC instructions. In brief, one-half ml of sterile water was added to each of two test tubes and the contents of each capsule were added to each

tube using a sterile pipet tip. The contents were thoroughly mixed and another 5 ml of sterile water was added to each test tube and mixed. These mixtures were allowed to incubate overnight at room temperature. The next day, the mixtures were transferred to Petri dishes containing Potato Dextrose Agar (PDA) (Becton, Dickinson and Company, Sparks, MD). Three plates for each strain were center inoculated and allowed to incubate in the dark at 30°C for two to three weeks. The spores were loosened by adding 2-3 ml of sterile water (repeated three times) and by brushing the mycelia with a sterile, bent glass rod. Spores of *P. expansum* 28883 and *P. expansum* 1117, ($2.09x10^7$ CFU/ml spore suspension and $2.69x10^7$ CFU/ml spore suspension, respectively) were collected in a 15-ml centrifuge tube and counted using a hemacytometer. The spore samples were stored at –80°C in 25% glycerol. Prior to use, spores of *P. expansum* 28883 and *P. expansum* 1117 were thawed at room temperature in a water bath and immediately transferred to the appropriate growth medium and incubated at 30°C.

3.3.2 Experiment 1: Ethylene and Patulin Production by *P. expansum* ATCC 1117 and ATCC 28883

To facilitate headspace gas sampling in the slant tubes used for this experiment, holes were drilled into the caps of 50-ml centrifuge tubes and Alltech, F-145 septum plugs were inserted. Twenty-five ml of horizontally aligned PDA or PDB was added to each tube. Spores of *P. expansum* ATCC 28883 and ATCC 1117 were added to 0.7% agar or additional broth to achieve a 10^5 CFU/ml spore suspension. The agar or broth in each 50-ml centrifuge tube was top inoculated with 4 ml of this mixture. Tubes were incubated at room temperature in darkness with the caps loosely threaded to allow gas exchange. Measurements of headspace gas for ethylene concentrations were conducted

every 20 to 28 hours for 10 days by gas chromatography. In order to measure headspace gas, tube caps were closed tight for 10-15 minutes to allow ethylene to accumulate. Headspace gas of each tube was then sampled by inserting a 26-gauge needle (attached to a 1 mL syringe) through the septum to obtain a 1 ml sample of headspace gas for ethylene measurement by gas chromatography (GC). Ethylene was determined in each tube in triplicate. This experiment was conducted to determine if *P. expansum* ATCC 1117 and ATCC 28883 produce ethylene when grown on solid PDA (1.5% agar), semi-solid PDA (0.7% agar), or liquid PDB (0% agar) media and, if so, to develop an approximate timeline for the ethylene synthesis by these fungi.

3.3.2.1 Extraction of Patulin from Media

The PDA samples were analyzed for patulin concentration by a combination of methods described by MacDonald, et al. (2000), Eisle and Gibson (2003), and Roze et al. (2004). Triplicate samples were obtained immediately after inoculation (day 0), daily on days 1-10, and finally on day 30 after inoculation and used for patulin quantification. First, the agar from each tube (including fungal mycelia) was cut into small pieces with a sterile knife and transferred to a 100-ml Erlenmeyer flask (Roze et al. 2005). Thrice, 25 milliliters of ethyl acetate were added to each flask and shaken for 1 minute (Roze et al. 2004). This mixture was allowed to sit for 8 to 12 hours and, again, shaken for 1 minute. Each time, the solvent was quickly removed using a Pasteur pipette and combined in a glass test tube (MacDonald, et al. 2000 and Roze et al. 2004). This solvent was evaporated to dryness under a gentle stream of nitrogen and reconstituted in 30 ml of pH 4 water (Prieta et al. 1993 and Rovira et al. 1993). Sample clean-up was conducted using

the solid phase extraction technique described by Eisle and Gibson (2003) and HPLC analysis was conducted.

Triplicate samples of each fungal strain grown in PDB also were analyzed for patulin concentrations immediately after inoculation (day 0), daily through day 10, and on day 25. First, the broth and mycelia from each tube was filtered through a Gooch crucible to collect the mycelia. The mycelia remaining in each Gooch crucible was thrice extracted with 25 milliliters of ethyl acetate added to the tube and shaken for 1 minute (Roze et al. 2004). This mixture was allowed to sit for 8 to 12 hours and, again, shaken for 1 minute. Each time, the solvent was quickly removed using a Pasteur pipette and combined in a glass container (MacDonald, et al. 2000 and Roze et al. 2005). This solvent was evaporated to dryness under a gentle stream of nitrogen and reconstituted in 30 ml of pH 4 water (Prieta et al. 1993 and Rovira et al. 1993). Samples were then subjected to solid-phase extraction of patulin followed by HPLC analysis.

3.4 Gas chromatography (GC) Quantification of Ethylene

Gas chromatography (GC) was performed using a 400 Series model gas chromatograph (Carle AGC, HACH Carle Chromatography, Loveland, CO). Ethylene concentrations in samples were calculated by comparison to known ethylene standards. These readings were performed in triplicate by taking 1-ml samples through septums placed in the cap of each tube using a 1-ml syringe.

3.4.1 Experiment 2: Patulin production in relationship to the action of endogenous and exogenous ethylene on solid and liquid* media plates

This experiment was conducted to determine the influence of endogenous and exogenous ethylene on patulin production by *P. expansum* in solid and liquid media,

respectively. The treatment design is presented in Table 10. This experiment was performed using a flow-through system consisting of eighteen 10-liter dessicators, each containing 10 Petri plates (100 mm x 20 mm) containing either PDA or PDB and center inoculated with 1 ml of 10^5 *P. expansum* spores. The flow-through system involved either 100 µL/L ethylene or air (containing no ethylene) being continuously flushed through the appropriate dessicator. The appropriate plates were treated with 1- MCP, the one time treatment at day 5, before they were placed in the dessicator (to compare directly with a concurrent study using apples), and others were continuously flushed with a 0.3 to 0.4 µL/L concentration of 1-MCP to control ethylene action of the fungi as it grew. The continuous 1-MCP treatment was dosed using a 1-MCP-releasing chemical mix [3000 µL/L, made fresh every other day; [stock of SmartFresh, Inc., Rohm and Hoas, Philadelphia, PA; 6.5 g plus 100 ml of water in a 980-ml jar (Roze et al. 2004)]. 1-MCP was delivered continuously using a syringe pump (KD Scientific, model 220, New Hope, PA). Appropriate dessicators contained granulated potassium permanganate ($KMnO_4$) and a carbon dioxide (CO_2) scrubber to absorb residual ethylene and residual CO_2, respectively. Cultures were grown in complete darkness. Ethylene concentrations in headspace gas were measured daily and plates were removed every 2 days for 10 days for patulin assays.

Ethylene measurements of head space gas in each dessicator were conducted daily by gas chromatography and the plates were analyzed for patulin presence using sample preparation techniques described by Prieta et al. 1993, Rovira, et al. (1993), MacDonald, et al. (2000), Eisle and Gibson (2003), and Roze et al. (2004). Patulin was extracted from the Petri plates containing potato dextrose broth using procedures similar to those

described for experiment 1. The mycelial diameter of fungal colonies on each plate was measured in centimeters using a ruler and recorded. The tubes containing solid media were heated for 30-45 minutes in a 90°C water bath. These samples were then filtered through Gooch crucibles to harvest mycelia. The mycelia and Gooch crucibles were dried in a 70°C oven for 12 hours, cooled in a dessicator, and weighed. They were then placed in the oven for two, subsequent, 30 minute periods, cooled, and weighed again. All three weights were recorded and averaged.

Table 10. Experiment 2 Treatment Design

Treatment	Inoculation	Ethylene	1-MCP (1-time injection)	1-MCP (Continuous)	Replicates
1	+	+	+	-	3
2	+	+	-	-	3
3	+	-	+	-	3
4	+	-	-	-	3
5	+	+	+	+	3
6	+	-	+	+	3

3.4.2 Solid-Phase Extraction

Solid-phase extraction (SPE) of patulin from apple juice was carried out using a syringe-cartridge method (Eisele and Gibson 2003). The SPE cartridge (Waters Oasis® HLB 60 mg, Milford, MA) was activated by applying 2-3 ml of water, followed by 2 ml of methanol and a final wash of 2-3 ml of water. Each sample (2.5 mL volume) was then applied to cartridges under vacuum. The cartridges were rinsed with 2.0 ml of 1.0% sodium bicarbonate solution (pH=8.3) followed by 2 ml of 1% acetic acid solution. One milliliter of 10% ethyl acetate in ethyl ether solution was then added to each cartridge to elute patulin. The eluates were collected in 15x45 mm vials, evaporated to dryness under

a stream of nitrogen and then reconstituted in 0.5 ml of 0.1% acetic acid. The vials were capped, vigorously mixed, and frozen for future analysis by HPLC.

3.4.3 High Performance Liquid Chromatography (HPLC)

Patulin was quantified using a Waters Corporation (Milford, MA) high performance liquid chromatograph (HPLC). The Waters system included two model 510 pumps, a model 717-plus auto-sampler, and a Photodiode Array detector (model 996, Milford, MA). An Alltech C_{18}, 5μm column (4.6 x 250 mm) was used to separate compounds. Each sample (50 μL injection volume) was subjected to isocratic elution for 30 minutes with 10% aqueous acetonitrile (ACN), followed by a 30 minute clean with 70% of 100% ACN and 30% of 10% ACN. The photodiode array detector collected the UV and visible light spectra from 190 to 800 nm to allow comparison of sample UV spectra to that of patulin standards. Chromatograms were analyzed using Waters' Millenium software. Patulin concentration was quantified by comparing UV absorbance at 276 nm to known patulin standards.

3.4.4 Statistical Analyses

For statistical analyses, SAS Version 9.1 was used (SAS Institute Inc., Cary, NC). In Experiment 1 (Tables 11-14), means and standard deviations were conducted for ethylene and patulin production by fungi grown on different media, but no statistical comparisons among media types or fungal strains were conducted due to individual sub-experiments being completed at different times.

In Experiment 2, data on $\log_{10}$ patulin concentrations were analyzed using PROC MIXED. Independent variables used in the model were the effects of ethylene

(exogenous or endogenous only), 1-MCP (none, once, continuous), the ethylene by 1-MCP interaction, time, and interactions between time with ethylene and 1-MCP. Experimental vessel (dessicator) was used as a repeated variable. An unstructured covariance procedure was used in the MIXED procedure. We evaluated the impact of ethylene and 1-MCP on the concentrations of patulin by comparing the appropriate means using two-sided t-tests in PROC MIXED in SAS. The value of 1 was assumed for samples having undetectable patulin (<4 μg/L patulin before log transformations to prevent undefined $\log_{10}$ values. Mycelia weights and mycelia diameters for Experiment 2 were analyzed using PROC MIXED and an autoregressive covariance matrix.

3.5 Results

3.5.1 Experiment 1: Production of Ethylene and by *P. expansum* 1117 and ATCC 28883 when Grown on Solid, Semi-Solid, and Liquid Media

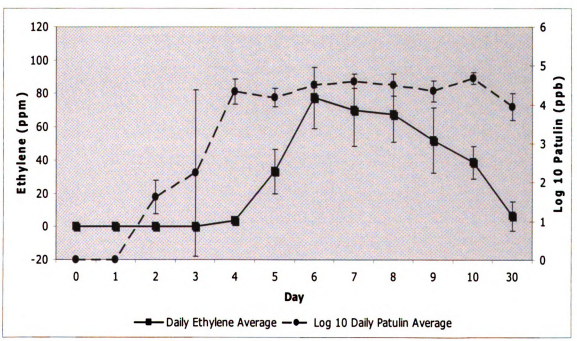
3.5.1.1 *P. expansum* 28883 on Solid Media

Data on ethylene and patulin productions by *P. expansum* 28883 grown on PDA (solid media) are presented in Table 11 and Figure 1. Patulin was first detectable 2 days after inoculation and approached its plateau by day 4. Ethylene production was zero or minimal until day 4, and reached peak production (77.5 ±18.5 μL/L) by day 6. Ethylene production was maintained at high levels through day 10 and still was being produced in significant concentrations on day 30 following inoculation.

Table 11. Patulin and Ethylene Production by *P. expansum* 28883 grown on Solid PDA Media

Day	Daily Patulin Average(μL/L)	Log_{10} Daily Patulin Average (μL/L)	Daily Ethylene Average (μL/L)
0	< 4	< 4	0.0025 ±0
1	< 4	< 4	0.0952 ±0.05
2	57 ±55	1.61 ±0.43	0.047 ±0.004
3	6373 ±10800	2.24 ±2.14	0.109 ±0.05
4	25591 ±14429	4.34 ±0.32	3.80 ±1.2
5	16986 ±8650	4.19 ±0.24	33.2 ±13.5
6	33245 ±6106	4.52 ±0.08	77.5 ±18.5
7	42724 ±16592	4.61 ±0.18	70.3 ±21.7
8	37048 ±25687	4.51 ±0.28	67.8 ±16.9
9	25514 ±14643	4.35 ±0.27	52.0 ±19.8
10	50067 ±18842	4.68 ±0.16	38.8 ±9.8
30	10949 ±8423	3.95 ±0.35	6.46 ±8.9

Figure 1. Patulin and Ethylene Production by *P. expansum* 28883 When Grown on Solid Media



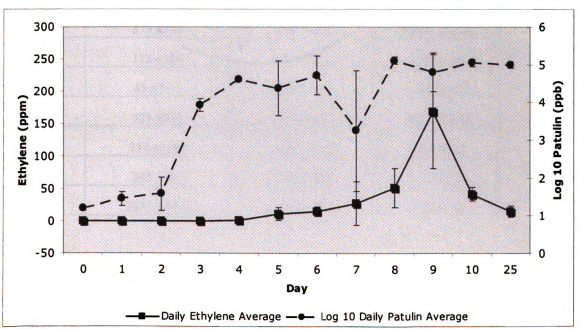
3.5.1.2 *P. expansum* 28883 on Semi-Solid Media

Data on ethylene and patulin productions by *P. expansum* 28883 grown on PDA (0.7% agar; semi-solid media) are presented in Table 12 and Figure 2. Since patulin was detectable immediately following inoculation, those samples on days 0 and 1 were treated as false positives, since no patulin should have been present at day 0 and at all times the patulin levels in semi-solid PDA was higher than that in solid PDA. As was observed when this strain was cultured on solid PDA, patulin production occurred before ethylene and peaked at day 4 (Figure 2). Ethylene production peaked at day 9. Relative patulin concentrations recovered from *P. expansum* 28883 grown on semi-solid PDA were slightly greater than for solid PDA. However, this appearance of increased recovery could be due to increased efficiency of patulin recovery from semi-solid versus solid PDA or due to an increase recovery of byproducts that coelute with patulin.

Table 12. Patulin and Ethylene Production by *P. expansum* ATCC 28883 When Grown on PDA Semi-Solid Media

Day	Daily Patulin Average(μL/L)	Log_{10} Daily Patulin Average (μL/L)	Daily Ethylene Average (μL/L)
0	16 ±0.005	1.19 ±0.06	0.0260 ±0.005
1	30 ±0.006	1.45 ±0.18	0.0491 ±0.006
2	53 ±0.02	1.58 ±0.44	0.0716 ±0.02
3	8701 ±0.02	3.92 ±0.17	0.107 ±0.02
4	40610 ±0.81	4.61 ±0.031	1.022 ±0.81
5	45458 ±10	4.38 ±0.73	10.8 ±10.01
6	73373 ±7	4.72 ±0.51	15.3 ±7.4
7	40716 ±34	3.25 ±1.60	27.8 ±33.7
8	130834 ±30	5.11 ±0.093	50.8 ±30.1
9	88669 ±11	4.79 ±0.52	169.3 ±87.9
10	113281±11	5.045 ±0.10	41.6 ±10.8
25	100401 ±9	5.00 ±0.09	15.08 ±8.61

Figure 2. Patulin and Ethylene Production by *P. expansum* ATCC 28883 When Grown on Semi-Solid PDA Media



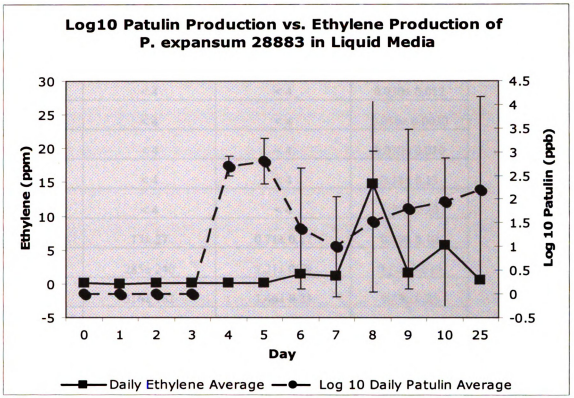
3.5.1.3 *P. expansum* 28883 on Liquid Media

Data on ethylene and patulin productions by *P. expansum* ATCC 28883 grown on PDB (liquid media) are presented in Table 13 and Figure 3. Patulin was first detectable 4 days after inoculation and approached its plateau by day 5. Ethylene production was zero or minimal until day 6, and reached a peak production (14.8 ±12.04 μL/L) by day 8. Production of both patulin and ethylene by *P. expansum* ATCC 28883 in liquid media were very attenuated relative to their productions when this organism was cultured on solid or semi-solid PDA.

Table 13. Patulin and Ethylene Production by *P. expansum* ATCC 28883 When Grown in Liquid PDB Media

Day	Daily Patulin Average(μL/L)	Log_{10} Daily Patulin Average (μL/L)	Daily Ethylene Average (μL/L)
0	< 4	< 4	0.055 ±0.0020
1	< 4	< 4	0.042 ±0.0039
2	< 4	< 4	0.065 ±1.32
3	< 4	< 4	0.060 ±0.0061
4	539 ±228	2.70 ±0.22	0.053 ±0.013
5	879 ±741	2.80 ±0.48	0.069 ±0.016
6	118 ±167	1.38 ±1.27	1.50 ±1.61
7	45 ±71.2	1.00 ±1.06	1.09 ±0.51
8	321 ±522	1.52 ±1.48	14.8 ±012.04
9	751 ±1202	1.80 ±1.68	1.62 ±0.77
10	243 ±302	1.95 ±0.92	5.75 ±9.04
25	2573 ±4018	2.19 ±1.98	0.57 ±0.41

Figure 3. Patulin and Ethylene Production by *P. expansum* ATCC 28883 When Grown in Liquid PDB Media



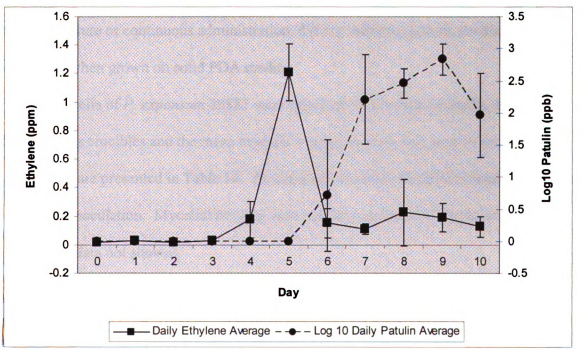
3.5.1.4 Patulin and Ethylene Production by *P. expansum* 1117 on Solid PDA Media

Data on ethylene and patulin production by *P. expansum* ATCC 28883 grown on PDB (liquid media) are presented in Table 14 and Figure 4. Patulin was first detectable 6 days after inoculation and reached its plateau by day 9. Ethylene production was zero or minimal until day 4, and reached a peak production (1.21 ±0.20 μL/L) by day 5. Unlike the results observed with *P. expansum* 28883, in this strain ethylene production occurred before patulin was detectable (Figure 4). Production of both ethylene and patulin by *P. expansum* ATCC 1117 were about 2 logs lower than were observed when *P. expansum* 28883 was grown on the same media.

Table 14. Patulin and Ethylene Production by *P. expansum* ATCC 1117 When Grown on Solid PDA Media

Day	Daily Patulin Average(μL/L)	Log_{10} Daily Patulin Average (μL/L)	Daily Ethylene Average (μL/L)
0	< 4	< 4	0.019± .0048
1	< 4	< 4	0.030± 0.012
2	< 4	< 4	0.016± 0.0030
3	< 4	< 4	0.030± 0.010
4	< 4	< 4	0.18± 0.13
5	< 4	< 4	1.21± 0.20
6	17± 27	0.71± 0.87	0.15± 0.10
7	285± 240	2.21± 0.70	0.11± 0.039
8	316± 162	2.46± 0.23	0.23± 0.23
9	766± 461	2.84± 0.24	0.19± 0.010
10	197± 275	1.96± 0.65	0.13± 0.072

Figure 4. Patulin and Ethylene Production by *P. expansum* ATCC 1117 When Grown on Solid PDA Media



3.5.2 Experiment 2: Patulin produced in relationship to the presence of endogenous and exogenous ethylene on solid and liquid* media

3.5.2.1 Solid Media

Since *P. expansum* 28883 was the strain to consistently produce high levels of patulin and ethylene in Experiment 1, it was the only strain used in Experiment 2. The effects of exogenous ethylene and 1-MCP treatment of patulin production by *P. expansum* when cultured on solid PDA media are presented in Table 15. Since no interaction between ethylene and 1-MCP on patulin production was detected, main effect least square means are presented. Averaged across all days and treatment levels, there was a significant (P = 0.02) effect of ethylene treatment on patulin production by *P. expansum* 28883 when grown on solid PDA media (Table 15). Plates exposed to 100 μL/L exogenous ethylene had significantly greater patulin production when compared to plates that were not exposed to exogenous ethylene. Treatment with 1-MCP, whether single exposure or continuous administration, did not influence patulin production by *P. expansum* when grown on solid PDA media.

Mycelia of *P. expansum* 28883 were collected by filtration of media through filtered glass crucibles and the mean mycelia weights on days 2, 6, and 10 following inoculation are presented in Table 16. As expected, mycelial weights increased with time after plate inoculation. Mycelial weights were not influenced by treatment with ethylene or 1-MCP (data not shown).

Mycelial colony areas on plates were measured on days 2, 4, 6, and 8 after inoculation and overall means averaged across treatments are presented in Figure 5.

Mycelia areas increased significantly ($P < 0.0001$) with time, but were not significantly influenced by ethylene or 1-MCP treatment (data not shown). Mycelia area was highly correlated with patulin production by *P. expansum* 28883 when grown on solid PDA media ($r = 0.80$; $P < 0.0001$)

Table 15. Patulin Concentrations (µg/L) of *P. expansum* 28883 Grown on Solid PDA Media and Treated with Ethylene and 1-MCP[1]

Day	Patulin Overall Mean (µg/L)	Log_{10} Patulin Mean (µg/L)	No Ethylene[a] (µg/L)	Ethylene[b] (µg/L)	MCP (-) (µg/L)	MCP (1x) (µg/L)	MCP (Con't) (µg/L)
4	13738 ± 1869	3.96 ± 0.06	3.66 ± 0.08	4.27 ± 0.08	4.00 ± 0.10	3.90 ± 0.10	3.98 ± 0.10
6	20087 ± 5951	4.04 ± 0.13	3.88 ± 0.19	4.19 ± 0.19	4.13 ± 0.23	4.03 ± 0.23	3.94 ± 0.23
8	53812 ± 14035	4.26 ± 0.17	4.05 ± 0.24	4.47 ± 0.24	4.07 ± 0.30	4.41 ± 0.30	4.29 ± 0.30
10	47072 ± 12862	4.35 ± 0.17	4.40 ± 0.23	4.30 ± 0.23	4.33 ± 0.29	4.52 ± 0.29	4.21 ± 0.29

[1] Least square means ± standard errors are shown.

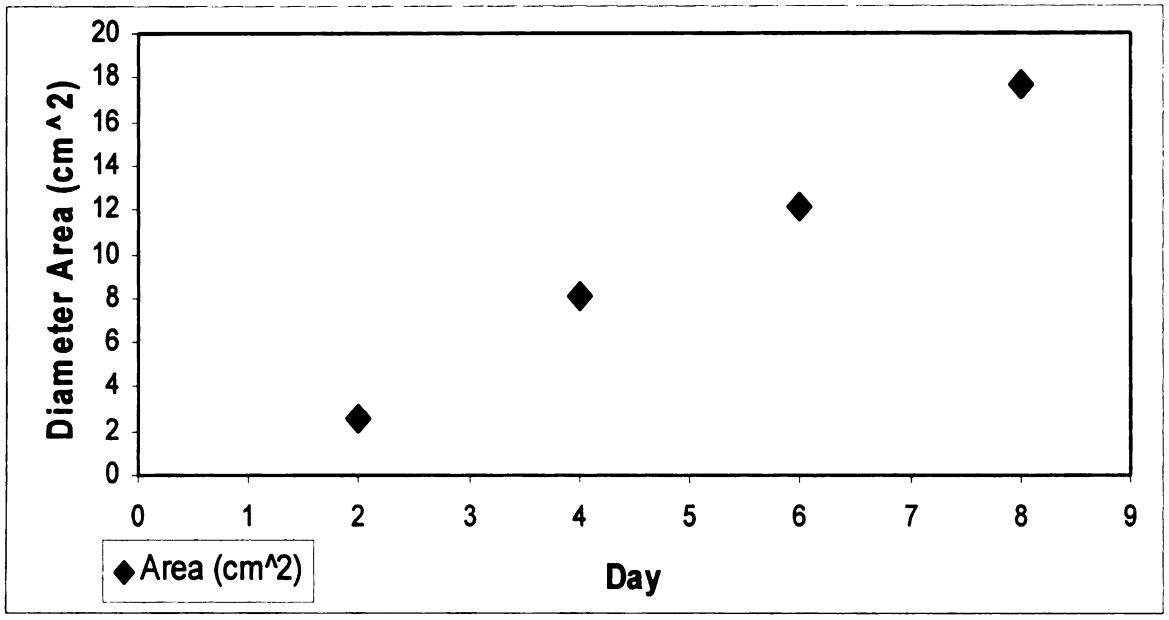
[ab] Main effect of ethylene ($P = 0.02$).

Table 16. Mycelia Weights (kg) of *P. expansum* 28883 Grown on Solid PDA Media and Treated with Ethylene and 1-MCP

Day	Average Weights (mg)
2	43.2^{a} ± 3.8
6	63.3^{b} ± 33.3
10	97.6^{c} ± 44.5

[a,b,c] Numbers with different superscripts (a,b,c) within a column significantly differ at $P \leq 0.05$.

Figure 5. Mycelia Areas (cm^2) in Solid Media Inoculated with *P. expansum* 28883 Treated with Ethylene and 1-MCP – Least Squares Means Averaged Across All Treatments



3.5.2.2 Liquid Media

When this experiment was conducted using PDB, too many plates contained undetectable patulin to allow for reliable statistical analysis. Therefore, these results are tabulated in the appendix to this dissertation (Appendix B), but are not discussed in this chapter.

3.6 Discussion

To improve safety of juice beverage products, in 2001, the U.S. FDA promulgated juice HACCP regulations to be implemented in all juice processing plants by January 2004 (FDA 2001). Also in 2001, FDA implemented additional regulations by limiting patulin concentrations in apple juice and cider, apple juice concentrate (at single strength), and apple sauce to 50 µg/L (FDA 2001). Thus, regulators are closely

scrutinizing patulin as a hazard of concern in apple juice products. The juice processing industry needs practical, useful methods to prevent or minimize the occurrence of patulin.

We hypothesized that ethylene regulation/manipulation could be a practical and economical solution to controlling patulin production in stored apples and, subsequently, in apple cider and juice produced from stored apples. Since patulin production generally occurs while apples are being stored, prevention in storage would likely be most beneficial to the juice and cider industry. Ethylene is a natural, gaseous plant hormone that has been found to inhibit aflatoxin biosynthesis in media and raw peanuts (Roze et al. 2004). Several strains of *P. expansum* have been reported, in non-peer-reviewed publications, to naturally produce large amounts of ethylene (Taiz and Zeiger 2002). It is not known how these ethylene levels related to the production of patulin by the mold. We conducted these experiments to determine if ethylene regulated patulin production by *P. expansum*. We also wanted to compare the effects of exogenous and endogenous ethylene on patulin production.

3.6.1 Experiment 1: Production of Ethylene by *P. expansum* ATCC 28883 on Solid, Semi-Solid, and Liquid Media and *P. expansum* ATCC 1117 on Solid Media

While it appears to be common knowledge that several species of Penicillium produce large amounts of ethylene (Taiz and Zeiger 2002), no studies were found that quantified these concentrations. When *P. expansum* ATCC 28883 was plated on solid, semi-solid, and liquid media, patulin production preceded ethylene production by the mold. Regardless of the media used, patulin consistently peaked at day 4, typically at least two days before ethylene production peaked. In fact, patulin production had declined somewhat during the times when ethylene production peaked and began to

decline. Patulin and ethylene concentrations when *P. expansum* ATCC 28883 was grown in liquid PDB media were much lower than those observed in solid and semi-solid media, which indicates that patulin production is significantly affected by host matrix.

Conversely, when *P. expansum* ATCC 1117 was cultured on solid PDA media, ethylene production preceded patulin production. Furthermore, *P. expansum* ATCC 1117 produced significantly less patulin and ethylene than *P. expansum* ATCC 28883, suggesting that considerable strain differences exist. Although this experiment proved that *P. expansum* is capable of producing large amounts of ethylene (albeit in a strain dependent manner) it did not give us a clear indication as to how patulin production is affected by endogenous ethylene. We observed in both strains of *P. expansum* that maximal production of ethylene and patulin occurred at different times. However, no clear hypothesis could be drawn about the influence of endogenous ethylene production on patulin synthesis.

3.6.2 Experiment 2: Patulin produced in relationship to the presence of endogenous and exogenous ethylene on solid media

Although there was no overall day effect on patulin production in liquid media, there appeared to be a tendency ($P = 0.06$) for exogenous ethylene exposure to accelerate patulin production by *P. expansum* 28883. Unlike experiments conducted with aflatoxin, ethylene did not inhibit or reduce patulin production in solid media inoculated with *P. expansum* (Sharma et al. 1985, Roze et al. 2004). In fact, patulin concentrations in Petri plates containing solid media inoculated with *P. expansum* ATCC 28883 were significantly greater in the presence of exogenous ethylene ($P = 0.0206$). We expected 1-MCP to promote patulin production (by blocking ethylene) in media in a manner similar

to that which occurred with aflatoxin studies (Roze et al. 2004). However, in this study we found no overall 1-MCP effect (P = 0.72) on patulin production nor on mycelia weight or mycelia area. It is possible that this lack of effect could be explained by the absorption of the 1-MCP by the $KMnO_4$ added to the containers to absorb ethylene, as it absorbs 1-MCP as well as ethylene.

Plates exposed to exogenous ethylene had high patulin concentrations at all times, which may be the main reason why no overall day effect was detected. Our finding that ethylene enhanced patulin concentrations was completely unexpected since exposure to exogenous ethylene in media and raw peanuts inhibited and/or reduced aflatoxin production by *Aspergillus parasiticus* (Roze et al. 2004, Sharma et al. 1985). This suggests that there is, in fact, a relationship between patulin production and the presence of ethylene.

Two differing conclusions could be drawn based on these results, 1) ethylene and patulin production by *P. expansum* might be unrelated or 2) ethylene promotes patulin biosynthesis by *P. expansum*. Since ethylene is a natural promoter of fruit softening and ripening, exposure to exogenous ethylene may simply increase fungal growth by facilitating mycelia penetration and, hence, indirectly increase patulin production. However, we consider the second explanation to be more likely. Although endogenous ethylene appears to be inversely related to patulin production in *P. expansum* ATCC 28883 and 1117, exogenous ethylene marginally promotes patulin production by *P. expansum* in solid media, despite the presence of continuous 1-MCP. Further research on potential mechanistic relationships between patulin and ethylene are warranted and it

would be useful to study the influence of 1-MCP and exogenous ethylene on patulin production in inoculated apples.

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4 Effect of fruit-produced ethylene and exogenous ethylene (100 μL/L) on the production of patulin in apples treated with 1-MCP and inoculated with *Penicillium expansum*

4.1 Abstract

Patulin is produced by molds including *Penicillium expansum* and is the most abundant mycotoxin in apples and apple juice. Production of patulin can be reduced under modified atmospheric storage conditions. Recent research shows that ethylene reduces aflatoxin production. The objective of this study was to determine whether fruit-produced ethylene or exogenous ethylene (100 μL/L) reduces the production of patulin in apples treated with 1-Methylcyclopropene (1-MCP), an ethylene receptor blocker, and inoculated with *Penicillium expansum*. Two studies were conducted to meet this objective. Both used freshly picked Red Delicious apples that were treated with 1-MCP, ReTain (an ethylene biosynthesis inhibitor), and then inoculated with *P. expansum*. The second experiment was expanded by the addition of sample vessels containing uninoculated apples to control for ethylene potentially produced by *P. expansum*. The apples were randomly assigned to 10-liter dessicators and then stored at ambient temperature either under 100 μL/L ethylene or 100% air. Apples from each container were obtained every two days after treatment and pressed to apple juice. Patulin in apple juice was quantified using solid phase extraction followed by high performance liquid chromatography. In the first experiment, concentrations of patulin in apple juice of *P. expansum*-inoculated apples increased exponentially from undetectable (< 4 μg/L) concentrations at day 0 to 3.44±0.10 μg/L after 16 days of storage, respectively ($P \leq 0.0012$). Ethylene treatment did not influence patulin concentrations in juice prepared

from apples in this experiment. In the second experiment juice from uninoculated fruit contained no detectable patulin. Concentrations of patulin in apple juice of *P. expansum* inoculated apples increased exponentially from undetectable (< 4 μg/L) concentrations at day 0 to 3.16±0.10 μg/L after 16 days of storage, respectively ($P \leq 0.0182$). Averaged across all time points, exposure of apples to 100 μL/L ethylene tended ($P \leq 0.065$) to increase the production of patulin in *P. expansum* inoculated apples. Neither ethylene nor 1-MCP and ReTain treatment affected patulin accumulation. We conclude that, contrary to our original hypothesis, exogenous ethylene treatment does not inhibit (and may actually promote) patulin synthesis by *P. expansum*.

4.2 Introduction

Patulin, a secondary fungal metabolite produced by several species of *Penicillium*, *Aspergillus*, and *Byssochlamys*, is the most common mycotoxin found in apples and apple juices (Wilson 1974, Lindroth 1980, Martins 2002). *Penicillium expansum*, which is commonly found in soil, is the primary source of patulin in fruit (Askar 1999, Jackson et al. 2003). Based upon its proven toxicity to animals and humans, an action level of 50 μg/kg was established by the U.S. FDA for patulin in apple juice, apple juice concentrate (at single strength), and apple sauce (FDA 2001).

Ethylene (C_2H_4) is a natural, gaseous compound that functions a plant hormone (Zeringue et al. 1990, Abeles et al. 1992, Savaldi-Goldstein and Fluhr 2000). The effects of ethylene on fruit ripening are well-characterized. Treatment causes lower ethylene emission by fruit and reduced incidence of core browning (Forsyth et al. 1969). However, in recent years it has become apparent that ethylene also functions as a

regulatory agent in the formation of secondary metabolites by certain species of fungi (Sharma et al. 1985).

For example, ethylene exposure has been shown to accelerate the softening of apples that can lead to rotting (Johnston et al. 2002). Preclimacteric McIntosh apples were studied under low (~6 ppm) and high (~1570 ppm) ethylene levels at 3.3°C for 189 days (Forsyth et al. 1969). Apples stored under low ethylene concentrations had higher firmness compared to apples stored at high ethylene levels, and this higher firmness continued for more than 7 days at room temperature (Forsyth et al. 1969). However, pH was lowered and soluble solids content was slightly increased in apples stored at low ethylene concentrations (Forsyth et al. 1969).

Aspergillus parasiticus and *Aspergillus nidulans* produce ethylene during the early growth phase on media, and the onset of aflatoxin biosynthesis is marked by the absence of ethylene evolution (Sharma et al. 1985). Sharma et al. (1985) demonstrated that ethylene may inhibit *Aspergillus* development and toxin biosynthesis by adding 2-chloroethyl phosphonic acid (CEPA), an ethylene-generating compound, directly to growth medium. These experiments demonstrate a tight relationship between ethylene presence and aflatoxin suppression in *A. parasiticus*, as well as between ethylene concentration and stimulation or inhibition of growth (Sharma et al. 1985). It is thought that the mechanism whereby ethylene reduces aflatoxin biosynthesis could be a receptor/sensor-mediated signaling pathway since 1-MCP, which has a structure similar to ethylene and blocks the ethylene receptors of higher plants, also promotes aflatoxin production by Aspergillus (Sharma et al. 1985, Serek et al. 1995, Roze 2004). Exogenous ethylene treatment prevents aflatoxin accumulation and sexual development

in *A. parasiticus* and *A. nidulans*, respectively (Roze 2004). The overall objective of this research was to determine the impact of ethylene exposure on patulin development by *P. expansum* inoculated on apples. We have previously demonstrated that patulin and endogenous ethylene production by *P. expansum* is inversely related, but that exogenous administration of ethylene to *P. expansum* 28883 stimulated patulin production.

The specific aims of this research were 1) to determine the effects of exogenous ethylene administration on patulin formation by *P. expansum* on Red Delicous apples, and 2) to assess the impact of 1-MCP on patulin production by *P. expansum* while growing on apples.

4.3 Materials and Methods

4.3.1 Experiment 1

The first experiment was conducted to determine the effect of exogenous ethylene treatment on patulin production by *P. expansum* on apples. Freshly picked Red Delicious apples (n=108) were treated with the ethylene inhibitor 1-Methylcyclopropene (1-MCP) overnight in three 5-gallon plastic containers. The apples were then dipped in ethanol and dual-hole punctured, using a wooden block containing two 3 mm metal pins that were spaced 1.8 cm apart. Before being used to inoculate fruit, the *P. expansum* used for this experiment was collected from decaying apples at MSU and propagated by vigorously mixing one moldy Honeycrisp apple in 200 mL of water. This solution was further diluted, serially, and plated. Pure ATCC cultures were not used due to time constraints and the desire to conduct immediate preliminary experiments. After culturing for 5-7 days, colonies were isolated and individually plated. The *Penicillium* classification was determined with the aid of a professional mycologist using a mycology

key (Barnett and Hunter 1998). Next, the apples were inoculated with *Penicillium expansum* using a 200 µl pipette tip. The apples were then randomly assigned to six dessicators (sixteen apples/dessicator) and stored at ambient temperature with a normal atmosphere or normal atmosphere plus 100 µL/L ethylene. The ethylene was produced by creating a vacuum in a gas cylinder, adding 540 mL of ethylene to the cylinder, and then adding compressed air to the cylinder until the final ethylene concentration was 100 µL/L. Rolling the tank mixed this combination and ethylene concentration was measured by gas chromatography. The gas phase of containers was maintained by the constant flow of gas from either the 100 µL/L ethylene or air tanks at a flow rate of 30 ml/min. Three dessicators were used for ethylene treatments and the other three dessicators were used for air treatments. Ethylene concentrations in the dessicators and the room were measured daily. Two apples from each container were randomly selected and removed after 8, 10, 12, 14, and 16 days of treatment, respectively, and pressed to obtain apple juice.

After sampling, apples were placed in freezer bags and frozen in a walk-in cooler (-20°C) until juicing. All equipment and utensils used for juicing were washed with water and ethanol and allowed to air dry between each sample. The apple samples were defrosted and cut in half on a cutting board using a sharp knife. The halves were then ground using a General Electric 4-speed food processor with a 450-Watt motor and then pressed using a potato ricer lined with cheesecloth into a beaker. The juice was poured into plastic culture tubes and capped and frozen until patulin was quantified using solid phase extraction (Eisele and Gibson 2003) followed by high performance liquid chromatography (MacDonald et al. 2000).

4.3.2 Experiment 2

Since preliminary results from Experiment 1 indicated that ethylene levels in the dessicators were not completely controlled in that study, a second experiment was designed. The treatment design used in this experiment (Table 17) included four treatments using apples inoculated with *Penicillium expansum* 28883 using a 2X2 factorial design of treatments having two levels of exogenous ethylene (100 uL/L ethylene; no ethylene) and two levels of 1-MCP and ReTain (treated; untreated). In addition, two treatments were included to test the effects of 1-MCP and ReTain on patulin and ethylene production of apples that were not inoculated with *P. expansum* and not treated with exogenous ethylene. Ethylene was prepared and dosed as described in Experiment 1. Apples were treated with ReTain (50 g/acre) four weeks before harvest. 1-MCP was also applied to apples as described in Experiment 1, with the exception that only half of the apples were treated. Inoculation with mold was the same as in Experiment 1, except that a commercial mold, *P. expansum* ATCC 28883 American Type Culture Collection (ATCC) (Manassas, VA), was used. After inoculation, the apples were then randomly assigned to 18 dessicators (sixteen apples/dessicator). Two apples from each container were randomly selected and removed after 3, 5, 7, 10, 12, 14, and 16 days of treatment, respectively, and pressed to obtain apple juice. Ethylene measurements in exit gas streams and patulin concentrations in juice produced from the treated apples were reported using gas chromatography and solid phase extraction and high performance liquid chromatography, respectively.

4.3.3 Preparation of Commercial Mold Cultures

A pure strain of *Penicillium expansum* ATCC 28883 mold culture was obtained as a freeze-dried culture from the American Type Culture Collection (ATCC; Manassas, VA). After the capsule was aseptically opened, the freeze-dried culture was rehydrated according to ATCC instructions. In brief, one-half ml of sterile water was added to each of two test tubes and the contents of each capsule were added to each tube using a sterile pipet tip. The contents were thoroughly mixed and another 5 ml of sterile water was added to each test tube and mixed. These mixtures were allowed to sit overnight in darkness at room temperature. The next day, the mixtures were transferred to Petri dishes containing Potato Dextrose Agar (PDA) (Becton, Dickinson and Company, Sparks, MD). Three plates for each test tube were center inoculated and allowed to incubate at 30°C for two to three weeks. The spores were loosened by adding 2-3 ml of sterile water (repeated three times) and by brushing the mycelia with a sterile, bent glass rod. Spores ($2.9x10^7$ CFU/ml spore suspension) were collected in a 15-ml centrifuge tube and counted using a hemacytometer. The spore samples were stored at –80°C in 25% glycerol. Prior to use, spores were thawed in a room temperature water bath and immediately transferred to the appropriate growth medium and incubated at 30°C.

4.3.4 Solid-Phase Extraction

Solid-phase extraction (SPE) of patulin from apple juice was carried out using a syringe-cartridge method (Eisele and Gibson 2003). The SPE cartridge (Waters Oasis® HLB 60 mg, Milford, MA) was activated by applying 2-3 ml of water, followed by 2 ml of methanol and a final wash of 2-3 ml of water. Each sample (2.5 mL volume) was then applied to cartridges under vacuum. The cartridges were rinsed with 2.0 ml of 1.0%

sodium bicarbonate solution (pH=8.3) followed by 2 ml of 1% acetic acid solution. One milliliter of 10% ethyl acetate in ethyl ether solution was then added to each cartridge to elute patulin. The eluates were collected in 15x45 mm vials, evaporated to dryness under a stream of nitrogen and then reconstituted in 0.5 ml of 0.1% acetic acid. The vials were capped, vigorously mixed, and frozen for future analysis by HPLC.

4.3.5 High Performance Liquid Chromatography (HPLC)

Patulin was quantified using a Waters Corporation (Milford, MA) high performance liquid chromatograph (HPLC). The Waters system included two model 510 pumps, a model 717-plus auto-sampler, and a model 996 Photodiode Array detector. An Alltech C_{18}, 5μm column (4.6 x 250 mm) was used to separate compounds. Each sample (50 μL injection volume) was subjected to isocratic elution for 30 minutes with 10% aqueous acetonitrile (ACN), followed by a 30 minute clean with 70% of 100% ACN and 30% of 10% ACN. The photodiode array detector collected the UV and visible light spectrum from 190 to 800 nm to allow comparison of sample UV spectra to that of patulin standards. Chromatograms were analyzed using Waters' Millenium software. Patulin concentration was quantified by comparing UV absorbance at 276 nm to known patulin standards.

4.3.6 Gas Chromatography (GC)

Gas chromatography (GC) was performed using a 400 Series model gas chromatograph (Carle AGC, HACH Carle Chromatography, Loveland, CO). Ethylene concentrations in samples were calculated by comparison to known ethylene standards.

These readings were performed in triplicate by taking 1-ml samples through septums placed in the cap of each tube using a 1-ml syringe.

Table 17. Treatment Design Used in Experiment 2.

Treatment	*P. expansum* Inoculation	100 µL/L Ethylene	1-MCP + ReTain	Replicates
1	+	+	+	3
2	+	+	-	3
3	+	-	+	3
4	+	-	-	3
5	-	-	+	3
6	-	-	-	3

4.3.7 Statistical Analyses

In Experiment 1, data on $\log_{10}$ patulin concentrations was analyzed using PROC MIXED. Independent variables used in the model were the effects of ethylene (exogenous or endogenous only), time, and interactions between time with ethylene. Experimental vessel (dessicator) was used as a repeated variable. An unstructured covariance procedure was used in the MIXED procedure. We evaluated the impact of ethylene on the concentrations of patulin by comparing the appropriate means using two-sided t-tests in PROC MIXED in SAS. The value of 1 was assumed for samples having undetectable patulin (<4 µg/L patulin before log transformations to prevent undefined $\log_{10}$ values.

In Experiment 2, data on $\log_{10}$ patulin concentrations was analyzed using PROC MIXED. Independent variables used in the model were the effects of ethylene (exogenous or endogenous only), 1-MCP (none, once), inoculation (inoculated, not inoculated), the ethylene by 1-MCP interaction, time, and interactions between time with ethylene, inoculation, and 1-MCP. Experimental vessel (dessicator) was used as a repeated variable. An unstructured covariance procedure was used in the MIXED

procedure. We evaluated the impact of ethylene and 1-MCP on the concentrations of patulin by comparing the appropriate means using two-sided t-tests in PROC MIXED in SAS. The value of 1 was assumed for samples having undetectable patulin (<4 μg/L patulin before log transformations to prevent undefined $\log_{10}$ values.

4.4 Results

4.4.1 Experiment 1: Ethylene, Air

Data on patulin concentrations in juice from *Penicillum*-infected apples treated with ethylene (100 μL/L) and air are presented in Table 18.

Concentrations of patulin in apple juice of *Penicillium*-inoculated apples increased exponentially from undetectable (< 4 μg/L) concentrations at day 0 to 3.44±0.10 μg/L after 16 days of storage, respectively ($P \leq 0.0012$). Patulin concentrations were not significantly influenced by exogenous ethylene treatment (P = 0.40), indicating that storage of apples in 100 ppm ethylene did not decrease the production of patulin in *Penicillium* inoculated apples.

Ethylene analyses of gases exiting the dessicators indicated that ethylene was being produced in the dessicators (data not shown). We could not attribute this ethylene production to 1) inadequate 1-MCP treatment of apples, or 2) ethylene production by *Penicillium*. Thus, Experiment 2 was designed to more carefully control for potential ethylene production by fruit or *P. expansum*.

Table 18. Experiment 1- Patulin Concentrations in Juice Prepared from Apples Treated with Ethylene (100 µL/L) and Air

Day	Mean	Ethylene[a]	Air[b]
8	1.35[a] ± 0.30	1.80 ±0.42	0.90 ±0.42
10	2.45[b] ±0.12	2.68 ±0.17	2.23 ±0.17
12	2.73[bc] ±0.18	2.57 ±0.26	2.88 ±0.26
14	3.22[cd] ±0.16	3.03 ±0.23	3.41 ±0.23
16	3.44[d] ±0.10	3.48 ±0.15	3.39 ±0.15

Least square means ± standard errors are shown.

[a,b,c,d]Numbers with different superscripts (a,b,c,d) within a column and row significantly differ at $P \leq 0.05$.

4.4.2 Experiment 2: Inoculation, Ethylene, Air, 1-MCP

Patulin concentrations ($\log_{10}$ ug/L) in juice from apples treated with ethylene and/or 1-MCP + ReTain in Experiment 2 are presented in Table 19. The results presented in Table 19 and the overall least mean square values of patulin concentrations in juice prepared from apples in treatments 1-4 of Table 17, as well as the main effect means for apples treated with 100 uL/L ethylene or no ethylene, and apples treated with 1-MCP + ReTain or untreated apples. Results for treatments 5 (MCP treatment, no inoculation, no ethylene treatment) and 6 (no treatment), which were not inoculated with *P. expansum*, are not presented because patulin concentrations were undetectable in the majority of juice samples pressed from these apples (data not shown).

Concentrations of patulin in apple juice pressed from *Penicillium expansum* inoculated apples (data not shown) increased exponentially ($P \leq 0.0182$) from undetectable (< 4 µg/L) concentrations at day 0 to 3.16 ± 0.10 µg/L after 16 days of storage. Averaged across all time points, treatment of apples with 100 ppm of exogenous ethylene tended ($P \leq 0.065$) to increase the concentration of patulin in juice pressed from

P. expansum-inoculated apples (Table 19 and Figure 6). There was no overall significant difference in patulin concentrations of juice prepared from apples treated with 1-MCP + ReTain versus those that were not ($P \leq 0.72$) (Table 19 and Figure 7). No significant interactions between ethylene and 1-MCP + ReTain treatment of apples were detected (data not shown).

Table 19. Patulin Concentrations (Log_{10} µg/L) in Juice Pressed from Apples Treated with Ethylene and 1-MCP + ReTain

Day	Overall Mean	Ethylene[d] (-)	Ethylene[e] (+)	1-MCP (-)	1-MCP (+)
5	1.99[a] ± 0.10	1.90 ± 0.15	2.08 ± 0.15	1.92 ± 0.15	2.07 ± 0.15
7	1.87[a] ± 0.21	1.69 ± 0.29	2.04 ± 0.29	1.79 ± 0.29	1.94 ± 0.29
10	2.56[b] ± 0.12	2.34 ± 0.16	2.78 ± 0.16	2.67 ± 0.16	2.45 ± 0.16
12	2.98[c] ± 0.088	2.96 ± 0.12	3.00 ± 0.12	2.93 ± 0.12	3.03 ± 0.12
14	3.19[c] ± 0.62	2.99 ± 0.088	3.37 ± 0.088	3.32 ± 0.088	3.05 ± 0.088
16	3.16[c] ± 0.10	3.02 ± 0.14	3.30 ± 0.14	3.26 ± 0.14	3.07 ± 0.14

Least square means ± standard errors are shown.

[a,b,c]Numbers with different superscripts (a,b,c) within a column and row significantly differ at $P \leq 0.05$.

[d,e]Ethylene Trend $P = 0.065$.

Figure 6. Effect of Ethylene on Patulin Concentrations in Juices Prepared from Apple infected with *P. expansum* 28883

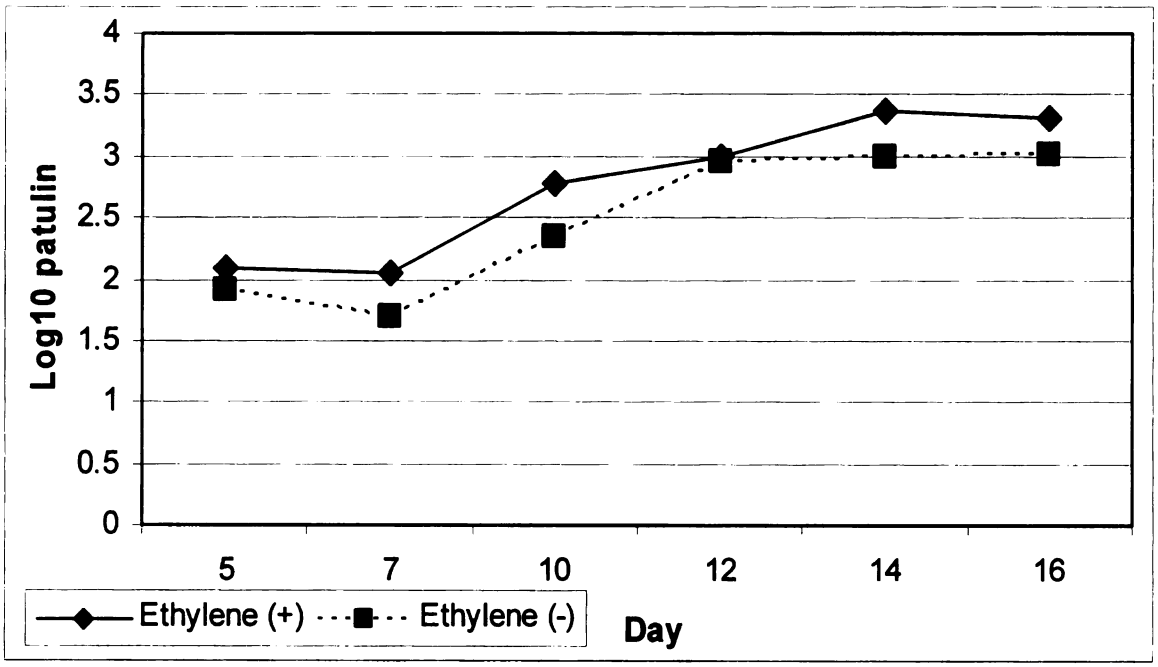
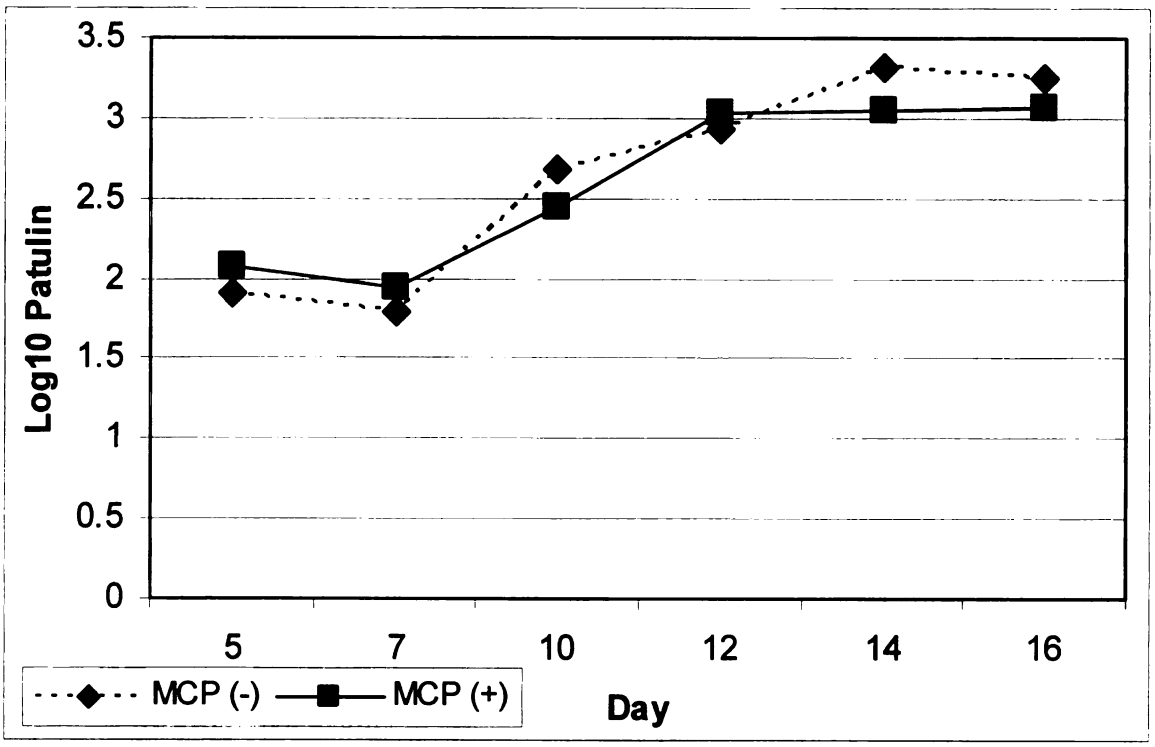


Figure 7. Effect of 1-MCP + ReTain on Patulin Concentrations in Juices Prepared from Apples infected with *P. expansum* 28883



4.5 Discussion

To increase juice safety, prevent foodborne illness, and enhance pathogen control, in 2001 the U.S. FDA promulgated juice HACCP regulations to be implemented in all juice processing plants by January 2004. In 2001, FDA also implemented additional regulations by limiting patulin concentrations in apple juice and cider, apple juice concentrate (at single strength), and apple sauce to 50 μg/L. The juice processing industry needs practical guidance to prevent or reduce the occurrence of patulin in juice beverages and other processed apple products. Based on its proven effectiveness in inhibiting aflatoxin production by Aspergillus (Roze et al. 2004 and Sharma et a. 1985), we hypothesized that ethylene treatment could be a practical and economical method to control patulin production in stored apples and, subsequently, in apple cider and juice produced from stored apples. Since patulin production generally occurs while fruit is being stored, prevention in storage would likely be most beneficial to the juice and cider industry.

Experiment 1 was a simple, preliminary study to examine the effects of exogenous ethylene administration (100 μl/L) on patulin production when compared to samples treated with only air. We chose 100 μL/L ethylene for these experiments because of its proven efficacy to reduce aflatoxin biosynthesis at this concentration (Roze et al. 2004). Contrary to our hypothesis, we observed no overall significant difference in patulin concentrations in juice prepared from apples stored in air versus those stored in 100 μl/L ethylene ($P = 0.40$). Our preliminary results from Experiment 1 indicate that patulin production by *Penicillium* is not subject to regulation by ethylene concentrations. However, we also were unable to completely inhibit ethylene production by apples (or

perhaps from *P. expansum*) with 1-MCP in this experiment. We developed the further hypothesis that the fungus was the source of ethylene when this study was repeated twice without completely inhibiting ethylene production, despite the use of 1-MCP. Therefore, it was concluded that these preliminary results were confounded and needed to be repeated, beginning with the verification that the 1-MCP was working properly using inoculation and lack of inoculation to allow calculation of endogenous ethylene production by the fungus.

Results from Experiment 2 confirmed that 1-MCP + ReTain treatment did not influence patulin concentrations in juice prepared from apples inoculated with *P. expansum*. This was unexpected to us since 1-MCP was found to promote aflatoxin production in media (Roze et al. 2004).

Our results from Experiment 2 do, however, suggest that treatment of apples with 100 µl/L ethylene tended ($P \leq 0.065$) to increase the production of patulin in *P. expansum*-inoculated apples. Although this result was unexpected, the finding that exogenous ethylene treatment may increase patulin production by *P. expansum* is in agreement with findings from experiments we conducted on patulin production by *P. expansum* 28883 when grown on PDA media (Chapter 3). This hypothesis was derived based upon similarities between aflatoxin and patulin, in that they are both mycotoxins that are synthesized via the polyketide pathway, producing 6-MSA, and have similar structures (Maggon et al. 1977, Tanenbaum and Bassett 1958, Zamir 1980).

This study was set up to further determine the effect of inoculation, to verify the proper operation of the 1-MCP/ReTain. By checking the ethylene levels daily using gas chromatography, it was found that the 1-MCP/ReTain was effective in inhibiting ethylene

production by the apples (See Appendix C). We also were able to confirm that *P. expansum* also was producing its own ethylene in the fruit, which agrees with our previous observations when culturing *P. expansum* on PDA media (Chapter 3). The average concentration of additional ethylene produced by apples that were not treated with 1-MCP + ReTain was 41.7 µl/L ethylene, which is significantly lower than the 100 µl/L exogenous ethylene used in this experiment (See Appendix C). It is unclear whether or not the concentrations of ethylene normally produced by untreated apples would actually promote patulin synthesis by *P. expansum*. This question should be addressed by future studies.

Ethylene is a hormone whose affects may be time sensitive. Continuous exposure to 100 µL/L ethylene tended to promote patulin production in apples inoculated with *P. expansum*. This long-term, continuous exposure may have influenced the biosynthesis of patulin in apples.

4.6 References

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5 Effect of trimming and culling on patulin concentrations in intact versus decayed apple tissues

5.1 Abstract

Patulin is the most common mycotoxin found in apples and apple juices. Because if its toxicity, the FDA has established that the patulin concentration of apple juice products should not exceed 50 ug/L. Additional research on quantitative apple-culling criteria necessary to produce juice meeting this criterion are required. Therefore, the objective of this study was to determine the effect of trimming of apples infected with *P. expansum* on patulin levels in normal-appearing and decayed apple tissue and in juice produced from two varieties of apples.

Two experiments were conducted to address the objective of this study. Experiment 1 utilized apples naturally infected. Experiment 2 utilized apples that we inoculated with *P. expansum* and stored for 3, 6, 9, 12, and 15 days. Infected apples were carefully trimmed to separate rot from normal-appearing tissue and juice obtained from each fraction tested for patulin by solid phase extraction and HPLC analysis.

In Experiment 1, juice prepared from high-quality apples that were free of decay with *P. expansum* did not contain detectable patulin. The average patulin concentration of juice produced from unculled naturally-infected apples was 918 μg/L. The proportion of rot in apples was not correlated to patulin concentrations in rot ($r = 0.054$). Juice prepared from normal-appearing tissue in five out of six replicates contained no detectable patulin, but the one replicate contained 110 μg/L of patulin.

In Experiment 2, there was no difference in patulin concentrations ($\log_{10}$ μg/L) in juice prepared from rotten tissue for Jonagold and Red Delicious apples when compared over a series of 15 days. Juice prepared from rotten tissue typically contained >1000 μg

patulin/L at all times beyond 6 days after inoculation. Juice prepared from normal-appearing tissue of both apple varieties always contained detectable patulin starting at 9 days after inoculation, but patulin concentrations in these samples never exceeded 100 µg/L. Trimming and culling rotten from normal-appearing apple flesh is effective in removing most patulin contamination from apples for juice production, although normal-appearing flesh of infected apples may still contain patulin concentrations in excess of regulatory limits.

5.2 Introduction

Patulin, a secondary fungal metabolite produced by several species of *Penicillium*, *Aspergillus*, and *Byssochlamys*, is the most common mycotoxin found in apples and apple juices (Wilson 1974, Lindroth 1980, Martins 2002). *Penicillium expansum*, which is commonly found in soil, is the primary source of patulin in fruit (Askar 1999, Jackson et al. 2003). Patulin causes gastrointestinal toxicity in rodents and also has mutagenic, neurotoxic, genotoxic and carcinogenic effects in rodents (Dickens and Jones 1961, Osswald 1978, Ciegler 1976, Reddy 1978, Reddy 1979, Lindroth 1980). Furthermore, patulin causes dermal and gastric irritation in humans and also suppresses the immune system of rodents, producing ulceration, congestion, and hemorrhagic lesions, especially in the gastrointestinal tract (Dickens and Jones 1961, McKinley et al. 1980ab, DeRosnay 1952, Dalton 1952). Based upon its toxicity profile, an action level of 50 μg/kg was established for patulin in apple juice, apple juice concentrate (at single strength dilution), and in beverages containing apple juice ingredients (FDA 2001).

Although thermal effects, charcoal treatment, ascorbic acid treatment, irradiation, filtration, centrifugation, and fermentation have been demonstrated to reduce concentrations of patulin in juice, trimming and culling of incoming fruit is the most effective method to reduce patulin in the resulting juice (Sands et al. 1976, Brackett and Marth 1979, Burroughs 1977, Bullerman and Hartung et al. 1975, Bissessur et al. 2001, Lovett et al. 1975). Trimming and culling has been shown to remove 93-99% of total patulin in fungus-rotted apples (Lovett et al. 1975). However, culling and trimming of fruit may not be a completely reliable means to control patulin concentrations, as a lack of visible rot does not necessarily indicate the absence of patulin in the fruit (Taniwaki

1992). Adequate culling of apples prior to juice preparation requires diligence and time and is often disregarded or poorly executed. Although over-ripened or rotting fruit can be removed, sorting based on visual observation is sometimes inadequate, resulting in patulin-contaminated finished products (Harwig et al. 1973).

Although previous studies on the effects of trimming and culling on patulin concentrations in apples have been conducted, several questions remain: 1) does removal or trimming of visible rot provide adequate control of patulin concentrations in the resulting juice? 2) are patulin concentrations in decayed or normal-appearing apple flesh influenced by apple variety? and 3) what is the relative patulin concentration in apple flesh decayed by *P. expansum*? It is important to determine the concentrations of patulin present in decayed and non-decayed portions of rotting apples, as well as the concentration of patulin in juice produced from these apples. This research will help determine apple-culling criteria necessary for processors to produce juice containing less than 50 μg patulin/L or juice. Therefore, the objective of this study was to determine the effect of trimming of apples infected with *P. expansum* on patulin levels in normal-appearing and decayed apple tissue and in juice produced from two varieties of apples.

5.3 Materials and Methods

Two different experiments were conducted to complete this study, one of which was comprised of three smaller trials. The first set of experiments involved Red Delicious apples that were naturally infected with *P. expansum* while the second experiment involved Jonagold and Red Delicious apples that were inoculated with *P. expansum* in the laboratory.

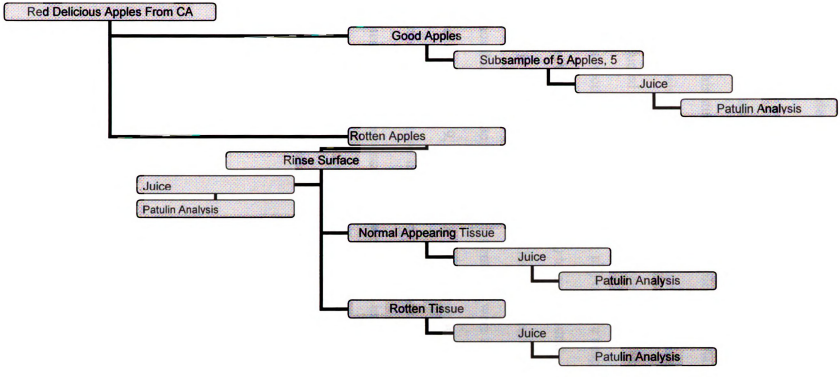
5.3.1 Experiment 1: Naturally Infected Apples

Apples used in this experiment were obtained from a commercial controlled atmosphere (CA) fruit storage facility in Grand Rapids, MI in July, 2005. Figure 1 outlines the steps used to fractionate apples and prepare samples for patulin analysis. First, red delicious apples were removed from CA storage and table sorted at the facility into intact, high quality fruit destined for the fresh market and culled fruit that included a significant proportion of damaged, moldy and decayed fruit. Lots of the high-quality fruit and the culled fruit were returned to the laboratory for further fractionation and sample preparation.

Five replicates (n=5 for each replicate) of high-quality fruit were randomly selected, homogenized, juice extracted, and tested for the presence of patulin by solid phase extraction and HPLC analysis. To extract juice, the samples were ground in a General Electric 4-speed food processor with a 450-Watt motor, and then pressed using a potato ricer lined with cheesecloth into a beaker. The juice was poured into plastic culture tubes, capped, and frozen until solid phase extraction and HPLC analysis was performed.

Among the culled apples, six replicates (n=10 for each replicate) of the red delicious apples were randomly selected, homogenized, and juice extracted as described above. Concurrently, 6 replicates of the same apples were selected and carefully trimmed to separate rot from normal-appearing tissue and juice was extracted from each fraction and tested for patulin. The rotten and normal-appearing tissue fractions were weighed to determine the percent rot for each replicate.

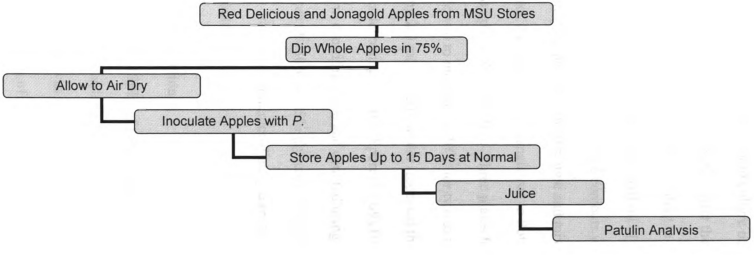
Figure 8. Experiment 1: Treatment of Apple Samples Obtained from Commercial Storage Facility



5.3.2 Experiment 2: Apples Inoculated with *Penicillium expansum*

This experiment was conducted using Jonagold and Red Delicious apples that were obtained from commercial sources and then inoculated with *P. expansum*. Figure 2 outlines the steps used to perform this study. Both varieties of apples were obtained from MSU Food Stores in August 2006 who received them from the State of Washington. *P. expansum* inoculation was conducted by wounding the apple surface using a two-hole (3 mm each) puncture wound and directly applying one drop (0.1 ml) of spore suspended (10^5 ug/mL water) *P. expansum* ATCC 28883 (Manassas, VA) to each wound. After inoculation, apples were stored in 6 (3 for each variety) 50-gallon plastic barrels at room temperature and normal atmosphere. Five apples from each container were randomly collected from each container on days 3, 6, 9, 12, and 15. A paring knife was used to remove the rotten area affected by *P. expansum* inoculation and separate it from the normal appearing tissue. These two different tissue types were placed in separate containers, then homogenized, pressed to extract juice, and analyzed for patulin using solid phase extraction followed by HPLC analysis.

Figure 9. Experiment 2: Treatment of samples for Trim/Cull Study



5.3.3 Preparation of Commercial Mold Cultures

Several steps were involved in the initial preparation of the freeze-dried capsule of *Penicillium expansum* ATCC 28883 mold culture obtained from the American Type Culture Collection (ATCC) (Manassas, VA). After the capsule was aseptically broken, the freeze-dried cultures were rehydrated according to American Type Culture Collection (ATCC) (Manassas, VA) instructions. One-half ml of sterile water was added to two test tubes and a capsule was added to each. The contents were thoroughly mixed and another 5 ml of sterile water was added to the test tube and mixed. This mixture was allowed to sit overnight. Next, the mixture was transferred to Potato Dextrose Agar (PDA) (Becton, Dickinson and Company, Sparks, MD). Three plates for each strain were center inoculated and allowed to incubate at 30°C for two to three weeks. The spores were loosened by adding 2-3 ml of sterile water (repeated three times) and by brushing the mycelia with a sterile, bent glass rod. Spores (2.09×10^7 CFU/ml spore suspension) were collected in a 15-ml centrifuge tube and counted using a hemacytometer. The spore samples were stored at –80°C in 25% glycerol. Before use, the samples were thawed in a room temperature water bath and immediately transferred to the appropriate growth medium and incubated at 30°C.

5.3.4 Solid-Phase Extraction

Solid-phase extraction (SPE) of patulin from apple juice was carried out using a syringe-cartridge method (Eisele and Gibson 2003). The SPE cartridge (Waters Oasis® HLB 60 mg, Milford, MA) was activated by applying 2-3 ml of water, followed by 2 ml of methanol and a final wash of 2-3 ml of water. Each sample (2.5 mL volume) was then applied to cartridges under vacuum. The cartridges were rinsed with 2.0 ml of 1.0%

sodium bicarbonate solution (pH=8.3) followed by 2 ml of 1% acetic acid solution. One milliliter of 10% ethyl acetate in ethyl ether solution was then added to each cartridge to elute patulin. The eluates were collected in 15x45 mm vials, evaporated to dryness under a stream of nitrogen and then reconstituted in 0.5 ml of 0.1% acetic acid. The vials were capped, vigorously mixed, and frozen for future analysis by HPLC.

5.3.5 High Performance Liquid Chromatography (HPLC)

Patulin was quantified using a Waters Corporation (Milford, MA) high performance liquid chromatograph (HPLC). The Waters system included two model 510 pumps, a model 717-plus auto-sampler, and a model 996 Photodiode Array detector. An Alltech C_{18}, 5μm column (4.6 x 250 mm) was used to separate compounds. Each sample (50 μL injection volume) was subjected to isocratic elution for 30 minutes with 10% aqueous acetonitrile (ACN), followed by a 30 minute clean with 70% of 100% ACN and 30% of 10% ACN. The photodiode array detector collected the UV and visible light spectrum from 190 to 800 nm to allow comparison of sample UV spectra to that of patulin standards. Chromatograms were analyzed using Waters' Millenium software. Patulin concentration was quantified by comparing UV absorbance at 276 nm to known patulin standards.

5.3.6 Statistical Analyses

For statistical analysis, SAS Version 9.1 was used (SAS Institute Inc., Cary, NC). In Experiment 1 (Table 20), means, standard deviation, and ranges of patulin concentrations (μg/L) were calculated for each fraction, Whole Apple (High-Quality), Whole Apple (Culled), Culled Apple Normal-Appearing Tissue, and Culled Apple Rotten

Tissue. Pearson Correlation Coefficients were calculated to determine the relationship between percent rot and patulin concentration or $\log_{10}$ patulin concentration in rotten tissue.

In Experiment 2, least square means of $\log_{10}$ patulin concentrations, as well as differences in varieties, days, containers, and tissue type, were analyzed using were obtained using PROC MIXED of SAS 9.1. We evaluated the impact of trimming, variety, day after inoculation, and their respective interactions on the concentration of patulin after using a $\log_{10}$ transformation of patulin concentrations. Container (replicate) was used as a repeated factor in this statistical analysis. One was added to undetectable (<4 μg/L patulin; 0.602 μg/L Log_{10} patulin) samples for log transformations to prevent unreadable error messages.

5.4 Results

Patulin concentrations in juice prepared from intact and fractionated apples in Experiment 1 are presented in Table 20. Juice prepared from high-quality apples culled by the packhouse manager did not contain detectable patulin. The lowest detectable concentration of patulin was 4 μg/L. The average patulin concentration of juice produced from whole culled apples was 918 μg/L, with a standard deviation of 1090 μg/L, and a range of patulin concentrations of 210 to 2522 μg/L. Normal-appearing apple tissue in five out of six replicates contained no detectable patulin. However, the replicate in which patulin was detected in normal-appearing tissue contained 110 μg/L of patulin. The mean and standard deviation of the patulin concentration in rotten tissue were 3553 μg/L and 3650 μg/L, respectively. The range of patulin concentrations in rotten tissues was 1529 to 10418 μg/L. The proportion of rot in apples (Figure 10) was not correlated to patulin concentrations ($r = 0.054$).

The proportion of rotten tissue in fractionated lots of apples was calculated for 5 of 6 the replicates (the sixth replicate rot yield was lost), and ranged from 25-50% rot. The patulin concentration detected in juice prepared from this rotten tissue ranged from 1529-10418 μg/L.

Table 21 shows the mean and range of concentrations of patulin detected in inoculated Jonagold apples. Patulin was detectable in juice from normal-appearing tissue beginning on day 9 after inoculation and patulin concentrations in juice from normal-appearing tissue ranged up to 65 μg/L. Patulin was detectable in juice prepared from rotten tissue at all times following fruit inoculation. Patulin concentrations in juice prepared from rotten tissue of Jonagold apples reached a plateau on day 9 following

inoculation at a mean patulin concentration of 2,951 ug/L. Trimming rotten tissue from normal-appearing tissue removed over 95% of patulin at all time points.

Table 22 shows the mean and range of concentrations of patulin detected in inoculated Red Delicious apples. Patulin was detectable in juice from normal-appearing tissue beginning on day 6 after inoculation and patulin concentrations in juice from normal-appearing tissue ranged up to 86 μg/L. Patulin was detectable in juice prepared from rotten tissue at all times following fruit inoculation. Patulin concentrations in juice prepared from rotten tissue of Red Delicious apples reached a plateau on day 12 following inoculation at a mean patulin concentration of 5128 μg/L. Trimming rotten tissue from normal-appearing tissue removed over 97% of patulin at all time points.

Figures 11 and 12 show the mean patulin concentrations over time in juice prepared from rotten and normal-appearing tissue of Jonagold and Red Delicious apples. In juice prepared from rotten tissue (Figure 11), there were not statistically significant differences in patulin concentrations between the two varieties at any time point after inoculation. In juice prepared from normal-appearing tissue (Figure 12), a significant difference ($P = 0.0025$) in apple variety was detected on day 9 after inoculation, but not at the other time points.

Table 20. Patulin Concentrations in Juice Prepared from Intact Apples, and Normal-Appearing and Rotten Tissue Fractionated from Apples

Among samples containing detectable patulin	Whole Apple (High-Quality)	Whole Apple with apparent rot (Culled)	Normal-Appearing Tissue of Fractionated Cull Apples	Rotten Tissue of Fractionated Cull Apples
Replicates	5	6	6	6
Total number of replicates containing detectable patulin (n)	0	6	1	6
Mean (µg/L)	ND*	918	110	3553
Standard Deviation (µg/L)	N/A	1090	N/A	3650
Range (µg/L)	ND	210 to 2522	<4 to 110	1529 to 10418

*ND = Not Detected, < 4 ug/L is the detection limit for this experiment. These samples were undetectable.

Figure 10. Relationship between Percent Rot to Patulin Concentration (µg/L) in rotten tissue (Experiment 1).

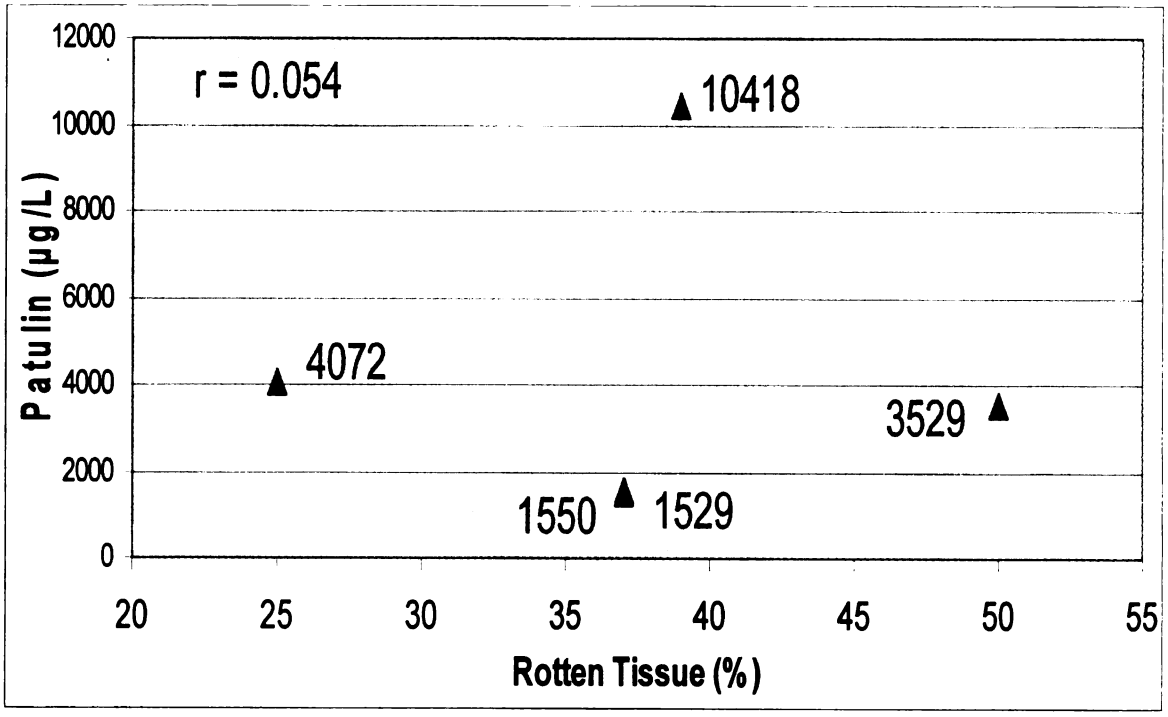


Table 21. Mean and Range of Patulin Concentrations in Juice Prepared from Normal-Appearing and Rotten Tissue of *P. expansum* Inoculated Jonagold Apples

Day	Mean in Normal-Appearing Tissue (Log_{10} µg/L)	Range in Normal-Appearing Tissue (µg/L)	Mean in Rotten Tissue (Log_{10} µg/L)	Range in Rotten Tissue (µg/L)	Patulin Removed by Trimming (µg/L) %
3	ND*	ND	1.66 ± 0.29	26 to 100	100
6	ND	ND	2.84 ± 0.21	226 to 1345	100
9	1.54 ± 0.12	18 to 51	3.47 ± 0.12	1566 to 5169	98.83
12	1.36 ± 0.17	9 to 53	3.35 ± 0.17	1177 to 3509	98.98
15	1.79 ± 0.15	56 to 65	3.11 ± 0.15	382 to 3249	95.21

*ND = Not Detected, < 4 ug/L is the detection limit for this experiment. These samples were undetectable.

Table 22. Mean and Range of Patulin Concentrations in Juice Prepared from Normal-Appearing and Rotten Tissue of *P. expansum* Inoculated Red Delicious Apples

ND = Not Detected, < 4 ug/L is the detection limit for this experiment. These samples were undetectable.

Day	Mean in Normal-Appearing Tissue (Log_{10} µg/L)	Range in Normal-Appearing Tissue (µg/L)	Mean in Rotten Tissue (Log_{10} µg/L)	Range in Rotten Tissue (µg/L)	Patulin Removed by Trimming (µg/L) %
3	ND*	ND	1.09 ± 0.29	ND to 37	100
6	0.30 ± 0.21	ND to 8	2.31 ± 0.21	92 to 382	99.02
9	0.69 ± 0.12	3 to 7	3.13 ± 0.12	1236 to 1463	99.64
12	1.50 ± 0.17	24 to 40	3.71 ± 0.17	3036 to 12385	99.38
15	1.80 ± 0.15	42 to 86	3.49 ± 0.15	2518 to 3778	97.96

Figure 11. Comparison of Patulin in Rotten Tissue by Apple Variety

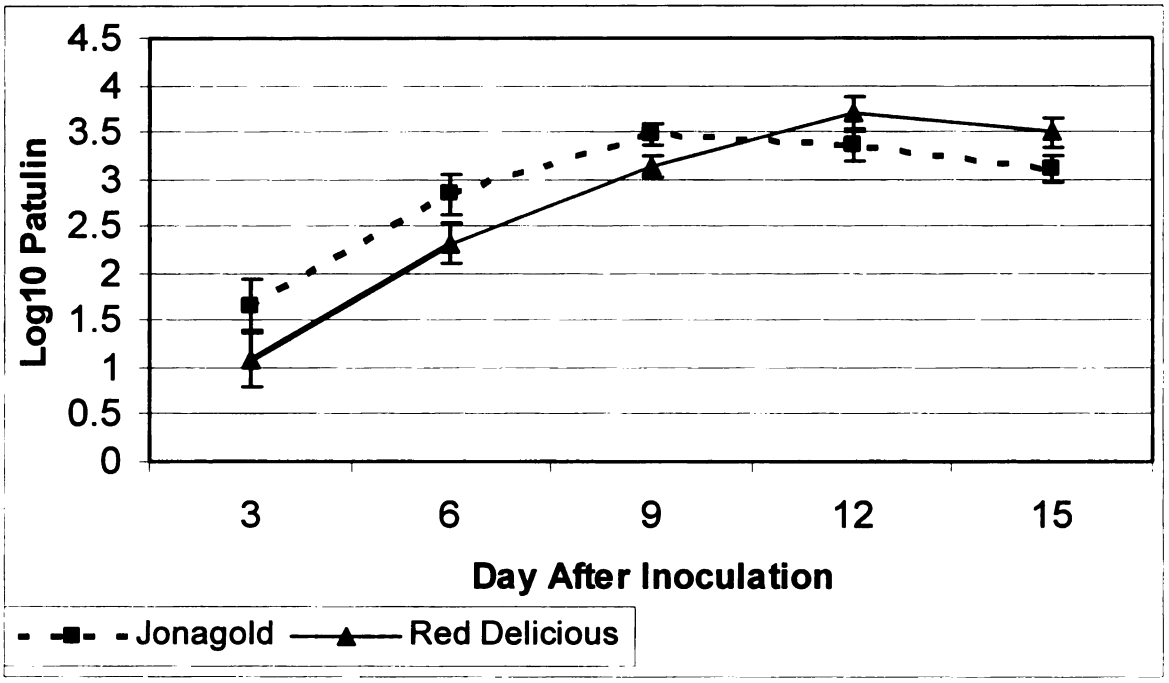
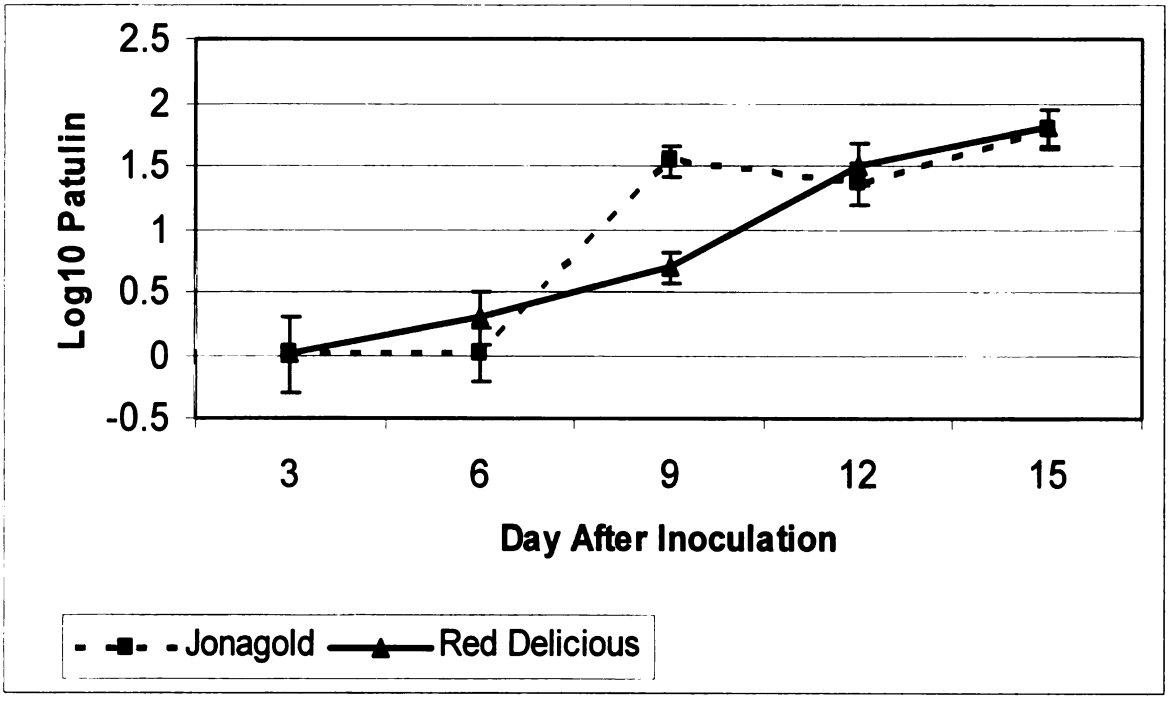


Figure 12. Comparison of Patulin in Normal Appearing Tissue by Apple Variety



5.5 Discussion

Our objectives in designing this experiment were to answer the three questions in the introduction. First, does removal or trimming of visible rot from apples provide adequate control of patulin concentrations in the resulting juice? Second, are patulin concentrations in decayed or normal-appearing apple flesh influenced by apple variety? Third, what is the relative patulin concentration in apple flesh decayed by *P. expansum*?

Previous research has addressed trimming of rot as a means to control patulin. Taniwaki et al. (1992) found that patulin did not migrate more than 1 cm into normal-appearing flesh of Ohio Beauty apples after all visible rot was removed. They did, however, note that the higher the percentage of rot allowed in apples used for juice, the higher the patulin concentrations of the resulting juice. They also noted that complete patulin removal based upon visibility, alone, did not exclude all patulin from juice.

In this experiment, we also found that trimming is quite effective in reducing patulin. However, apple samples containing only normal-appearing tissue still contained patulin levels near or at the legal limit of 50 ug/L after only 9 days following inoculation with *P. expansum*. While the inoculation level (10^5 CFU/ml) used in this experiment may not commonly occur in nature, *P. expansum* is naturally present in soil and has been found to inoculate apples in a natural environment (Harwig et al. 1973). Lovett et al. demonstrated a 93-99% patulin reduction in trimmed apples (1975). Our research demonstrated that trimming removed 97% of patulin from juice prepared from naturally-decayed Red Delicious apples (Experiment 1) and <95% of patulin from Red Delicious and Jonagold apples that were artificially inoculated with *P. expansum* in the laboratory.

Lovett et al. (1975) suggested that variety of apple may play a role in patulin production, since rotten tissue in Red Delicious apples contained less patulin (<5 μg/ml) than rotten tissue in Golden Delicious and McIntosh apples (42.5-117.5 μg/ml) when inoculated with *P. expansum* 1071 and 1172 in their experiment. No significant differences were due to apple variety found in this study in patulin concentrations of juice prepared from rotten or normal-appearing tissue of Red Delicious and Jonagold apples. However, we did note that, after inoculation, Jonagold apples decayed at approximately twice the rate of Red Delicious apples. Hence, we found that apple variety may affect rate of decay, but not patulin concentration in rot.

The majority of moldy apples in which only *P. expansum* is isolated will contain patulin. Moldy apples that do not contain patulin tend to be inoculated with other molds (Harwig 1973). Patulin concentrations in juice prepared from culled apples in Experiment 1 and trimmed, rotten tissue and some normal appearing tissue after only three days of inoculation in Experment 2 were well above the FDA established maximum residue levels (MRLs) of 50 μg/L. These data represent the importance of a trim, cull, or patulin prevention method for all varieties of apples. The Juice HACCP Alliance (2002) recommends that "No more than 1% by weight rot after culling" remain in apples for juicing in order to control patulin. Based on the maximal concentrations of patulin in apple rot determined in this research, the suggested critical limit of 1% rot allowed in apples destined for apple juice may not be completely sufficient. Based on the maximal patulin concentrations extracted from apple rot in these experiments, it appears that culling to 0.4% rot may be necessary to maintain juice <50 μg/kg patulin. However,

further studies should be conducted to ensure that the remaining pulp used for animal feed will also contain <50 μg/kg patulin when this percentage rot is used.

The proportion of rot in apples used in Experiment 1 was not correlated with patulin. This lack of correlation could be due to some rot in experiment 1 being due to molds other than *P. expansum* which do not produce patulin. However, rot caused by *P. expansum* is likely to have a uniformly high concentration of pautlin based on our results in Experiment 2.

Commericial facilities use different methods to prevent the entry of patulin into their samples. Scrubbers with hard bristles, pressure washing, and culling by humans are popular methods used in small and large cider mills and commercial, juice facilities. Although there is no guarantee that patulin will not remain in normal-appearing tissue, scrubbers with hard bristles to remove decayed flesh are probably effective for patulin prevention. Human culling could be just as effective, depending on the diligence of the individual performing the task. However, it is important to note that normal-appearing apple tissue from carefully trimmed fruit in this experiment still yielded juice having >50 μg patulin/L. It is reasonable to presume that manual culling and trimming by humans in typical juice processing facilities would be less stringent than was conducted in this experiment. Culling must be done with extreme care and attentiveness, which makes it a tiring job that is often either poorly conducted or entirely neglected.

When the 2002-2004 Michigan cider mill results were combined (N=493), patulin was detected (≥4 μg/L) in 18.7% of all cider samples with 2.2% of samples having patulin concentrations above the legal limit of 50 μg/L. Approximately 23.3% of all cider and juice samples (N=159) collected from retail grocery stores in 2005-2006

contained detectable (≥4 μg/L) patulin, with 11.3% samples having patulin concentrations above the legal limit of 50 μg/L. These statistics are indicative of a serious problem in the apple juice and cider industry, not only in Michigan cider mills, but also internationally with the analysis of national brands. The industry is in serious need of effective, easily implemented methods of patulin control in juice from whole apple fruit and concentrate. Although the industry does implement trim and cull methodology, it, apparently, is not completely reliable.

Although trimming is effective, culling appears to be a more reliable method of patulin control, since no matter how meticulous the trimmer, normal-appearing tissue may still contain patulin levels above 50 μg/L. Therefore, we highly recommend that the industry cull rotten apples from samples destined for juice until patulin production can be prevented or significantly reduced in controlled atmosphere storage. Since the industry, due to historical and economically efficient practices, will likely continue to trim in order utilize as much fruit as possible, we recommend that trimming be done to a maximum of 0.4% rot (weight/weight).

5.6 References

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6 Summary, Conclusions and Future Research

Patulin is produced by mold species such as *Penicillium expansum* and is the most abundant mycotoxin in apples and apple juice. Because of its toxicity, the FDA has established that patulin concentration of apple juice products should not exceed 50 μg/liter (U.S. FDA, 2001, 21 CFR Part 120, Fed. Register 66:6138-6202). Michigan ranks as one of the top three apple producing states with approximately 30% of Michigan apples processed into cider. However, the levels of patulin in Michigan apple cider have not been systematically determined. Production of patulin can be reduced under modified atmospheric storage conditions. Recent research shows that ethylene reduces the biosynthesis of aflatoxin, another mycotoxin, by *Aspergillus spp.* The relationship between ethylene production by *P. expansum* and the onset of toxin production has not been examined.

Our surveillance research indicates that the majority of apple cider produced by Michigan mills in 2002-2004 contained no detectable patulin. Patulin was detected (≥4 μg/L) in 18.7% of all cider mill samples with 11 (2.2%) samples having patulin concentrations above the legal limit of 50 μg/L. A greater percentage of cider samples obtained from mills using thermal pasteurization contained detectable patulin (28.4%) when compared to mills using ultraviolet-light irradiation (13.5%) or no pathogen reduction treatment (17.0%). Among juice and cider samples obtained from retail grocery stores (N=159), 23% contained detectable patulin, with 18 (11.3%) samples having patulin concentrations above the legal limit of 50 μg/L. Some apple juice samples obtained from retail grocery stores had exceptionally high patulin concentrations, ranging up to 2700 μg/L.

Timing of patulin and ethylene production by *P. expansum* was inversely related for *P. expansum* ATCC 28883 and 1117. Patulin production in Petri plates containing PDA inoculated with *P. expansum* ATCC 28883 was significantly increased by the presence of exogenous ethylene, but there was no effect of 1-MCP on patulin production. These results confirm that *P. expansum* produces ethylene and suggests an inverse relationship between ethylene and patulin production.

Concentrations of patulin in apple juice produced from *P. expansum* 28883 inoculated apples increased exponentially from undetectable (< 4 μg/L) concentrations at day 0 to 3.16±0.10 μg/L after 16 days of storage. Averaged across all time points, exposure of apples to 100 μL/L ethylene tended to increase the production of patulin in *P. expansum* inoculated apples. Therefore, exogenous ethylene treatment does not inhibit (and may actually promote) patulin synthesis by *P. expansum*.

The average patulin concentration of juice produced from unculled naturally-infected Red Delicious apples was 918 μg/L. The proportion of rot in apples was not correlated to patulin concentrations in rot. Juice prepared from normal-appearing tissue in five out of six replicates contained no detectable patulin, but the one replicate contained 110 μg/L of patulin. Juice prepared from rotten tissue typically contained >1000 μg patulin/L at all times beyond 6 days after inoculation. Juice prepared from normal-appearing tissue of both apple varieties always contained detectable patulin starting at 9 days after inoculation, but patulin concentrations in these samples never exceeded 100 μg/L. We highly recommend that the industry cull rotten apples from samples destined for juice until patulin production can be prevented or significantly reduced in controlled atmosphere storage. Since the industry, due to historical and

economically efficient practices, will likely continue to trim in order utilize as much fruit as possible, we recommend that trimming and culling be done such that no more than 0.4% rot (weight/weight) remains in apples pressed for juice.

Several important questions remain to be studied and should be addressed in future research, 1) does "core rot," also known as "moldy core," contain patulin, 2) what are optimal control atmosphere conditions to minimize patulin contamination, 3) by what mechanism does exogenous ethylene enhance patulin production by P. *expansum*, 4.) what is the natural variance in ethylene production and patulin production across different strains of *P. expansum*, and 5) what is the effect of inoculum level on rot and patulin production.

7 Appendices

Appendix A

General Cider Information

7.1 General Cider Information

Michigan is one of the largest fresh apple and apple products producers in the United States, producing and processing an average of 764 million pounds of apples 2000-2003 (Doores 1983-1984, Michigan Apple Association 2004, Michigan Agricultural Statistics 2003, Thede 2004). Only the states of Washington and California produce more fresh apples and apple products, respectively (Doores 1983-1984). An average of 207.5 million pounds (27%) of juice and cider were donated to juice in 2000-2003 (Doores 1983-1984, Michigan Apple Association 2004, Michigan Agricultural Statistics 2003, Thede 2004). The Michigan apple industry grossed $250,457,000 pounds in 2003 (Michigan Agricultural Statistics Service 2004).

7.1.1.1 Processing Steps

Several steps are required in the processing of cider. After the apples are harvested, they are generally placed in storage, washed, and inspected. This inspection is generally used to separate apples to be sold wholly or as juice from dropped, decayed and damaged apples, a process known as culling (Beaudry 2005, Thede 2004). Culling is essential since damaged fruit is more likely to carry pathogens and are more susceptible to chemical hazards, such as patulin. This may be followed by a grinding step (Thede 2004). A hydraulic press is then used to further crush the ground samples into juice (Thede 2004). The juice is filtered and held in a tank where it may or may not undergo a 5-log reduction step (Thede 2004). This is followed by bottling and refrigerated storage (Thede 2004).

7.1.1.2 HACCP

There are an estimated 16,000 to 48,000 juice related illnesses per year in the United States (FDA 2001). Most of these are suspected to be caused by contaminated fruit and pathogenic microorganisms, especially in unpasteurized juice (Besser et al. 1993, FDA 2001). These facts and several recalls led the FDA to address foodborne illness and hazards related to juice products (1997, Thede 2004). A warning statement on juice product labels was required in 1998 if they were not processed using HACCP or a lethality step that would lead to a 5-log reduction of the pertinent pathogens (FDAb, Thede 2004). Furthermore in 1998, FDA stated an intent to require juice processors to develop and implement HACCP systems (Thede 2004). To heighten juice manufacturers' knowledge, educational programs on juice safety and HACCP were established (Thede 2004). The FDA gave the juice industry three years to fully execute HACCP programs in 100% juice beverage products and in retail establishments or businesses that make and sell juice to other businesses (2001). Large processors were to implement programs by January 2002, followed by small and very small processors in January 2003 and January 2004, respectively (1998a). Although HACCP does not directly apply to growing, harvesting, and transporting of fruits and vegetables, key personnel are encouraged to implement Good Agricultural Practices (GAPs), Current Good Manufacturing Practices (CGMPs), and Sanitation Standard Operating Practices (SSOPs) (Dingman 1999, Codex 2003).

Appendix B

Patulin and Ethylene Production when Grown in *P. expansum* 28883 in Liquid PDB Media

7.1.2 Patulin produced in relationship to the presence of endogenous and exogenous ethylene on solid and liquid media

Table 23. Patulin Concentrations (Log_{10} µg/L) of *P. expansum* 28883 in Liquid Media Treated with Ethylene and 1-MCP

Day	Mean	Ethylene* (-)	Ethylene* (+)	1-MCP (-)	MCP (+)	MCP (Con't)
6	$0.4432^{a} \pm 0.173$	0.0886 ± 0.244	0 ± 0.244	$.0732 \pm 0.299$	0.5979 ± 0.299	0 ± 0.299
8	$1.0587^{ab} \pm 0.103$	1.35 ± 0.145	0.7627 ± 0.145	0.780 ± 0.178	1.6247 ± 0.178	0.772 ± 0.178
10	$0.4702^{ac} \pm 0.116$	0.0200 ± 0.164	0.7403 ± 0.164	0.107 ± 0.201	0.4523 ± 0.201	0.851 ± 0.201
12	$2.1469^{d} \pm 0.100$	2.19 ± 0.142	2.1064 ± 0.142	2.16 ± 0.174	2.3015 ± 0.174	1.98 ± 0.174

Least square means ± standard errors are shown.

[a,b,c]Numbers with different superscripts (a,b,c) within a column and within pathogen reduction measures or year of processing significantly differ at $P \leq 0.05$.

Table 24. Overall Mycelial Weights (g) and Diameters (cm) in Liquid Media Inoculated with *P. expansum* 28883 Treated with Ethylene and 1-MCP

Day	Average Weight (g)
4^{a}	0.120 ± 0.0075
6^{a}	0.134 ± 0.0075
10^{b}	0.159 ± 0.0075

[a,b]Numbers with different superscripts (a,b,c) within a column and within pathogen reduction measures or year of processing significantly differ at $P \leq 0.05$.

Appendix C

Supplemental Data on Ethylene Production by Apples for Chapter 4

Table 25. Ethylene production (outflow – inflow in closed vessels) of Red Delicious Apples variously treated with *P. expansum*, ethylene, and 1-MCP.

Treatment	Overall Difference Ethylene Mean (μL/L)
Ethylene, 1-MCP, Inoculated	1.50 ± 5.71^{a}
Ethylene, No 1-MCP, Inoculated	14.08 ± 5.7^{ab}
No Ethylene, 1-MCP, Inoculated	2.13 ± 5.71^{ab}
No Ethylene, No 1-MCP, Inoculated	19.42 ± 5.71^{b}
No Ethylene, 1-MCP, Uninoculated	0.16 ± 5.71^{a}
No Treatment	41.54 ± 5.71^{c}

Least square means ± standard errors are shown.

[a,b,c]Numbers with different superscripts (a,b,c) within a column significantly differ at $P \leq 0.05$.