リンゴの貯蔵温度のP. expansumの生育とパツリン産生への影響

誌名	マイコトキシン
ISSN	02851466
著者名	東原,圭亮 竹内,正彦 板東,誠治 三枝,曜介 宮川,浩美 後藤,哲久
発行元	マイコトキシン研究会
巻/号	59巻1号
掲載ページ	p. 7-13
発行年月	2009年1月

Effect of storage temperature on the growth of *Penicillium expansum* and its production of patulin in apples

Keisuke HIGASHIHARA [*1], Masahiko TAKEUCHI [*2], Seiji BANDOH [*1,3], Yosuke SAEGUSA [*1], Hiromi MIYAGAWA [*1] and Tetsuhisa GOTO [*1,4]

[*1] Faculty of Agriculture, Shinshu University (Minami Minowa, Nagano, 399-4598)
[*2] Agricultural Technology Institute of Nagano Farmers' Federation (Suzaka, Nagano, 382-0084)

Abstract

The relationship between the effect of storage temperature, the growth of *Penicillium expansum* and its production of patulin on apple were studied. To prevent fungal spoilage and patulin production it was demonstrated that apples needed to be stored just below 0 °C. It was also shown that once fungal decay had commenced, it spread relatively quickly and that patulin was produced, even when apples were stored at +1 °C. However, when apples were stored at +5 °C or below there was some time lag before decay and the onset of patulin formation. Therefore it was concluded that it is very important to store apples at +5 °C or less as soon as possible after harvest.

Key words : patulin, *P. expansum*, storage temperature, decay, control

(Received: September 22, 2008, Accepted: January 17, 2009)

Introduction

Patulin is one of the toxic secondary fugal metabolites produced by *Aspergillus clavatus, Penicillium expansum, Byssochlamys nivea* and *some others*[1, 2]. Patulin contamination is often found in apples and apple products[1, 3-5]. Patulin was first discovered as an antibiotic in 1942 and has an antibiotic effect on both Gram negative and Gram positive bacteria[6]. Patulin displays a range of toxic effects that include DNA damage[7], mutagenicity[8], neurotoxicity[9] and immunosuppression[10]. Therefore Codex Alimentarius has recommended a patulin content of less than 50 μg/kg in apple products.

Apple can become contaminated with patulin in the orchard before harvest [11] but the incidence of patulin contamination increases during storage. The apple harvest commences in late summer and continues until late autumn depending on the cultivar. The processing of apples to juice and the production of other apple products commences immediately after harvest and may continue until early spring. Patulin producing fungi, such as *P. expansum*, can grow and produce patulin on apples during this storage period. When apples are stored at ambient temperatures the onset of spoilage by

[*3] Current address: Tama Laboratory, Japan Food Research Laboratories (6 Nagayama, Tama-shi, Tokyo 206-0025)
[*4] Corresponding Author

P. expansum can be very rapid. To prevent patulin contamination in apples and apple products, they are often kept in cold storage before processing. However, the cold storage of apples is energy consuming and costly. So it is very important to understand the optimal conditions for apple storage to prevent patulin contamination of apples and apple products. In this study, we studied the relationship between storage temperature and time for patulin formation in apples, using the most popular cultivar (Fuji) that is used for apple processing as well as some other cultivars which are harvested earlier than Fuji and that are also used for processing.

Materials and methods

Chemicals and fungi Patulin, 4-hydroxy-4*H*-furo-[3,2-c]pyran-2(*6H*)-one, standard was purchased from Sigma-Aldrich (MI, USA) and Biopure (Tulln, Austria). Patulin standard stock solution was made in ethyl acetate and stored at -20 °C. To make the patulin working solution, an aliquot of patulin stock solution was put into a small amber glass vial and dried under a gentle stream of nitrogen gas, then it was dissolved with acetic acid-water pH 4 (water adjused its pH to 4 by acetic acid). 5-HMF, 5-hydroxymethylfurfural, was purchased from Acros (Geel, Belgium). Potato dextrose agar (PDA) medium was purchased from Difco (MD, USA). All other chemicals were either HPLC grade or GR grade and purchased from Kanto Chemical Co. Ltd (Tokyo, Japan) and used without further purification. Water was purified by Mill-Q system (Millipore, MA, USA).

Four strains of *P. expansum* were cultured of PDA slant medium for 2 to 3 weeks. Patulin production by these fungi on apples was tested and confirmed using the method described later. *Penicillium expansum* Pa977 and Pa948, isolated by Dr. K. Suzaki at National Institute of Fruit Tree Science were donated from Dr. Tabata (Tokyo, Japan).

Preparation of samples A spore suspension of the mold was prepared using 0.1 % Tween 20 solution. The surface of the apple was wiped with 80 % ethanol and spores were inoculated into the apple about 1~2 mm below the surface using a needle. Apples were individually wrapped in aluminum foil then incubated at the designated temperature in the dark. The temperature of incubators and apple samples during cultivation was monitored using a Center 309 data logger thermometer (MK Sientific, Tokyo, Japan). After incubation, the decayed area of the apple was measured.

For patulin analysis, the apple was divided into 9 pieces using an apple cutter. From in our previous study, patulin concentrations in juice and pressed residue were almost same (data not shown) the piece(s) which decay was observed was pressed to prepare juice for patulin analysis. For each study 5 to 6 apples were used.

Extraction of patulin from apple juice Patulin was extracted from the samples using a method with minor modifications from AOAC-OMA method 995.10[12, 13]. Also for some samples, a more thorough clean-up method[14] was applied.

For the AOAC-OMA 995.10 methods, five grams of sample were measured into a 18 x 180 mm glass test tube and extracted twice with 10mL ethyl acetate (10 mL x 2). The ethyl acetate phases were combined and washed with 2 mL of 1.5 % sodium carbonate solution. The aqueous layer was re-extracted with 5 mL of ethyl acetate. The ethyl acetate phases were combined and dried with 1 g of anhydrous sodium sulphate. The ethyl acetate phase was transferred to a 100 mL recovery shaped

glass flask and 5 % volume of acetic acid-ethyl acetate (2:98, v/v) was added. It was concentrated to 1~2 mL by a rotary evaporator (RE111, Sibata, Tokyo, Japan) at 40 °C. The ethyl acetate layer was transfered into 8 mL glass amber vial and evaporated to dryness under a gentle nitrogen stream at 40 °C. Residues were dissolved with 500 μL ethyl acetate for LC and LC-MS analysis. For Kawamoto's method[14], the same amounts of sample were extracted by ethyl acetate and a silica gel column was used for the clean-up.

LC and LC-MS analysis Patulin was analyzed by a single quadropole type LC-MS (Shimadzu LCMS-2010EV) with a UV detector. For quantitative purpose, UV absorption was mainly used and MS detection was mainly used for confirmation.

The LC portion of the LCMS-2010EV system was a model LC-2010CHT liquid chromatograph system (Shimadzu). Ten μL of sample was injected. Patulin was separated by an ODS column (Synergi Hydro-RP 80A, 2.0 mm i.d. x 250 mm, Phenomenex) at 40 °C. The mobile phase was a mixture of water and acetonitrile (95:5, v/v) with a flow rate was 0.2 mL/min. Patulin was also monitored by UV absorbance detector at 276 nm.

Electrospray ionization (ESI) was used to introduce the sample to the MS and the patulin was detected at m/z 153, 125 and 109 by selected ion monitoring mode. ESI was carried out at a spray voltage of -3.5 kV, temperatures of 200 °C and 250 °C were applied to the Curved Desolvation Line (CDL) and heat block and nitrogen was used as a nebulizer gas at a flow rate of 1.5 L/min. The voltage of CDL and detector were used at 15.0 V and 1.5 kV. Samples were analyzed in negative ion mode.

Results & Discussion

LC and LC-MS analysis As shown in Fig. 1, patulin was detected around 13.5 min by both UV and MS and clearly separated from HMF. The lower detection limit (S/N=3) by UV absorbance for standard patulin was 0.01 ng and the quantitation curve was linear from 0.05 to 40 ng. Patulin was

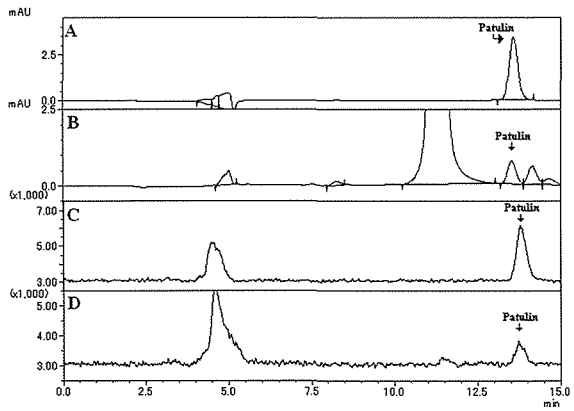

Fig. 1 Chromatograms of patulin standard and apple extract
A: patulin standard (2 ng, 276 nm), B: apple extract (20 μg/kg, 276 nm)
C: patulin standard (2 ng, m/z=153), D: apple extract (20 μg/kg, m/z=153)

Table 1. Patulin production and decay caused by *P. expansum* at 20 °C on Fuji apple

Isolates	days	decay (mm)	Patulin (μg/kg per apple)
NRRL6069	8	50	600
IFO-7100	8	68	300
Pa948	8	65	2800
Pa977	8	60	10

successively analyzed in the range from 2 to 40,000 μg/kg in apple juice. The lower detection limit (S/N=3) of patulin by m/z=153 was ca 0.1 ng and the quantitation curve was linear from 0. 5 to 40 ng. Patulin was successively analyzed in the range from 20 to 40,000 μg/kg in apple juice.

Decay and patulin formation at 20 °C Apples are harvested mainly in autumn and in some cases apples are left at ambient temperature before being processed. Therefore in this study we inoculated four strains of *P. expansum* into Fuji apple and stored them at 20 °C. Within 2 days after inoculation, the decay on the apples spread approximately 6 mm per day. As shown in Table 1, after 8 days, the area of decay was 50 - 68 mm in diameter and the patulin concentration per apple reached up to 2800 μg/kg, about 60 times more than the action level set by the Japanese Government (50 μg/kg). Also when these four isolates were inoculated on other cultivars of apples (Akibae, Sinano gold and Tugaru) the diameter of decay was. 28 - 40 mm and 30 - 4000 μg/kg of patulin were detected after 8 days. This result shows the importance of cooling apples rapidly after harvest to prevent the growth of *P. expansum* and patulin formation.

Effect of storage temperature for* P. expansum *growth and patulin contamination on apple

When Fuji apples were inoculated with *P. expansum* and stored at -2.5 and -5 °C, no decay occurred but the apples were frozen and lost their economic value (Table 2). When *P. expansum* was inoculated on a PDA plate it grew well at +2.5 and +5 °C but when inoculated into apples at these temperatures it took 2-5 weeks for decay to be visible (Fig. 2). However, once the decay became visible it increased appromately 1 mm diameter per day and patulin was produced in the apple (Fig. 2). Similar results were obtained even at +1 °C (Fig. 3). Apples inoculated with *P. expansum* and then stored for 2 months at 1 °C produced areas of decay 14 - 45 mm in diameter and significant amounts of patulin were detected in the apples (Table 3).

At -0.5 °C, *P. expansum* grew on the PDA plate, but when inoculated into apples very slow growth, after some time lag, was observed at -0.5 °C. Eighty three days after inoculation decay became 2 to 7 mm in diameter (Fig. 4), but patulin was not detected from these apples (Table 2). At -1.0 °C, *P. expansum* (strains NRRL6069, Pa977 and Pa948, but not IFO-7100) grew on the PDA plate after some time lag, but no growth or patulin production was observed on the apples.

These results show that in order to maintain the commercial value of apple it is important to store them at just below 0 °C so as to prevent spoilage by *P. expansum* and the formation of patulin.

Conclusion

Fungal spoilage by *P. expansum* and the formation of patulin can occur very quickly on apples if

Table 2. Patulin production and decay caused by *P. expansum* on Fuji apple

Temp. (°C)	20	5	2.5	1	-0.5	-1	-2.5	-5
Storage period (days)	8	60	60	63	83	63	*b	*b
Decay (mm)	61	70	55	23	4	2	Frozen*c	Frozen
Patulin concentration (μg/kg/apple) *a	900	1300	350	300	N.D.	N.D.	*d	*d

*a: Average of four *P. expansum*
*b: Storage period is not applicable because frozen
*c: Apple was frozen and no mold growth was obserbed.
*d: No analysis was done because apple was frozen
N.D.: Not Detected (less than 0.5 μg/kg at the portion analyzed)

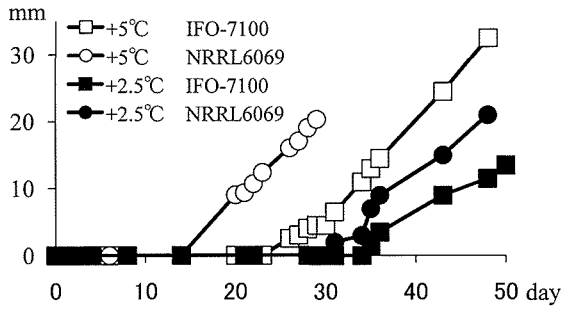

Fig. 2 Growth of decay caused by *P. expansums* on apple at +2.5 and +5 °C

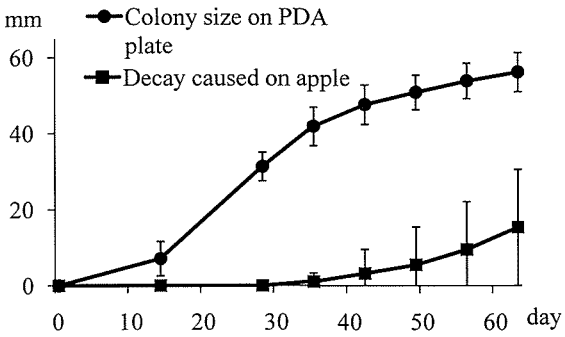

Fig. 3 *P. expansum* growth on PDA and apple at +1 °C
Average of four *P. expansum* isolates (One PDA plate and 5 apples for each isolates)

they are stored at +20 °C and also at +5 °C, the temperature at which apples are usually kept in cold storage. Our study showed that it is important to store apples just below zero centigrade to prevent fungal spoilage and patulin contamination so as to maintain the commercial value of apple. However, the commercial storage of apples at such a low temperature is energy consuming and expensive. Our results also showed that when apples are stored at +1 °C (Table 2), there is still the potential for fungal spoilage and patulin production by *P. expansum*. However, at low temperature even at +5 °C (Fig. 2), there is some time lag before the decay on apple by *P. expansum* starts. Therefore to prevent the formation of patulin it is very important to store apples in cool conditions as soon as possible after

Table 3. Patulin production and decay caused by *P. expansum* at 1 °C on Fuji apple

Isolates	days	decay (mm)	Patulin (μg/kg per apple)
NRRL6069	63	17	13
IFO-7100	63	45	1000
Pa948	63	15	140
Pa977	63	14	53

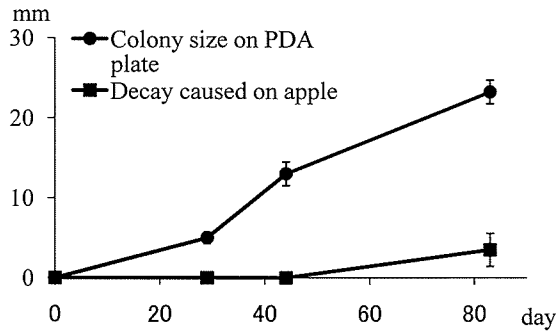

Fig. 4 *P. expansum* growth on PDA and apple at -0.5 °C
Average of four *P. expansum* isolates (One PDA plate and 5 apples for each isolates)

harvest.

The authors thank Mr. A. E. Buckle for his suggestions during the preparation of this manuscript. This research was supported by Research Project for Utilizing Advanced. Technologies in Agriculture, Forestry and Fisheries, the Ministry of Agriculture, Forestry and Fisheries of Japan.

References

1) Frank, H. K.: Ann. Nutr. Alim, **31**, 459-465 (1977)
2) Ciegler, A., Mycopathologia, **65**, 5-11 (1978)
3) Scott, P.M., Miles, W.F., Toft, P., Dobe, J.G.: J. Agric. Food Chem., **20**, 450-451 (1972)
4) Spadaro, D., Ciavorella, A., Frati, S., Garibaldi, A., Gullino, M.L.: Food Control, **18**, 1098-1102 (2007)
5) Tabata, S.: Mycotoxins, **58**, 129-135 (2008)
6) WHO: In WHO Food Additives Series 35, WHO, Geniva, (1996)
7) Liu, B.-H., Yu, G.-Y., Wu, T.-S., Li, S.-Y., Su, M.-C., Wang, M.-C., Shih, S.-M.: Toxicol. Appl. Pharmacol., **191**, 255-263 (2003)
8) Schumacher, D.M., Metzer, M., Lehmann, L.: Arch. Toxicol., **79**, 110-121 (2005)
9) Sabater-Vilar, M., Maas, R.F.M., Bosschere, H.D., Ducatelle, R., Fink-Gremmels, J.: Mycopathologia, **158**, 419-426 (2004)
10) Escoula, L., Thomsen, M., Bourdiol, D., Pipy, B., Peuriere, S., Roubinet, F.: Int. J. Immunopharmacol., **10**, 983-989 (1988)

11) Suzaki, K., Ito, T., Kanematsu, S.: Mycotoxins, **58**, 137-141 (2008)
12) AOAC International: AOAC Official Method 995.10 Patulin in apple juice., In: Horwitz, W. (ed.), Official Methods of analysis of AOAC International, 18th Ed., AOAC International, Gaithersburg, MD, USA (2005)
13) Brause, A. R., Trucksess, M.W., Thomas, F. S., Page, S.W.: J. AOAC, **79**, 451-455 (1996)
14) Kawamoto, Y., Bandoh, S., Higashihara, K., Miyagawa, H., Goto, T.: World Mycotoxin J., **1**, 59-65 (2008)

リンゴの貯蔵温度の *P. expansum* の生育とパツリン産生への影響

東原圭亮，三枝曜介，宮川浩美，後藤哲久：信州大学農学部（399-4598　長野県上伊那郡南箕輪村 8304）
竹内正彦：㈱長野県農村工業研究所（382-0084　長野県須坂市須坂 787-1）
板東誠治：信州大学農学部（399-4598　長野県上伊那郡南箕輪村 8304）
現　㈶日本食品分析センター多摩研究所（206-0025　多摩市永山 6-11-10）

リンゴの貯蔵温度と，かび加害，パツリン蓄積の関係を検討した．その結果，リンゴでの菌の加害とパツリン産生を抑制するには 0 ℃ 程度の温度が必要であり，また一度菌の繁殖が始まると 1 ℃ でも加害が進行しパツリンが蓄積した．一方，5 ℃ 以下でリンゴを貯蔵したとき，菌による加害が始まるまでに温度によって一定の時間がかかることが示された．このことは，収穫後のリンゴを速やかに低温に置くことが，加害防止の観点からは非常に重要であることを示すものであった．

キーワード：パツリン，*P. expansum*，貯蔵温度，腐敗，制御