Effects of Plant Volatile Compounds on Fungal Growth and Patulin Production of *Penicillium expansum* in Apple Juice

植物が生産する揮発性化合物によるリンゴ果汁における *Penicillium expansum* の生育およびパツリン産生に対する影響

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Contents

LIST OF ABBREVIATIONS USED

6-MSA	6-methylsalicylic acid
ABC	ATP-binding cassette
acetyl-CoA	acetyl-coenzyme A
ATCC	American Type Culture Collection
β-Tub	β-tubulin gene
CAC	the Codex Alimentarius Commission
CAR	Carboxen
cDNA	complementary deoxyribonucleic acid
CFDA	5(6)-carboxyfluorescein diacetate
CFU	colony-forming units
Ct	cycle time
DON	deoxynivalenol
DVB	divinylbenzene
E double bond	trans double bound
EtOH	ethanol
EU	European Union
GC-MS	gas chromatography/mass spectrometry
GSH	glutathione
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
IARC	the International Agency for Research on

	Cancer
IDH	isoepoxydone dehydrogenase gene
JECFA	the FAO/WHO Joint Expert Committee on Food Additives
λEm	emission wavelength
λEx	excitation wavelength
LC/MS	liquid chromatography/mass spectrometry
malonyl-CoA	malonyl-coenzyme A
MeCN	acetonitrile
MeOH	methanol
MIC	minimum inhibitory concentration
msas	6-methylsalicylic acid synthase gene
MSD	mass selective detector
NaCl	sodium chloride
NIST	National Institute of Standards and Technology
N-terminals	amino-terminals
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
PDMS	polydimethylsiloxane
peab1	ATP-binding cassette transporter gene
PI	propidium iodide; 3,8-Diamino-5-[3-(diethylmethylammonio)

	propyl]-6-phenylphenanthridinium diiodide
PTFE	polytetrafluoroethylene
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
SD	standard deviation
SPME	solid phase micro-extraction
Tween 80	polyoxyethylene sorbitan monooleate
UV	ultraviolet

Chapter 1

General Introduction

Food safety is one of the most important concerns for both the food industry and consumers. Foods are sometimes contaminated with toxic fungal secondary metabolites called mycotoxins, whose contamination poses a great threat to human health all over the world. There are several types of mycotoxins with different structures, such as aflatoxin, DON (deoxynivalenol), ochratoxin, and patulin.

Aflatoxins are polyketide secondary metabolites produced by *Aspergillus flavus*, *A. parasiticus*, *A. tamarii* and *A. nomius*. They are the most notorious mycotoxins because of their high carcinogenicity for human and animals.[1,2] Their contamination of corns, peanuts, pulse, spices, and tree nuts is not only a serious health hazard but also an economic problem worldwide. Aflatoxins B_1, B_2, G_1, and G_2 are major aflatoxins produced by the fungi mentioned above (Fig. 1-1). Because of the risks of acute hepatic disorder and liver cancer that the ingestion of aflatoxins presents, many countries have strict regulation values for aflatoxins, and in Japan, the requirement for the contamination level of aflatoxin in all foods is less than 10 μg/kg as total amount of aflatoxin B_1, B_2, G_1, and G_2.

DON is a terpenoid mycotoxin produced by *Fusarium* sp., the cause of Fusarium head blight of wheat. Based on its structural features, DON and its analogs are categorized in the B group of trichothecene

mycotoxin (Fig. 1-2).[3] DON has strong, acute toxicity and it contaminates corn and wheat. Therefore, in the EU, the regulation values for DON contamination are established against individual foods; e.g., 0.2 mg/kg for processed cereal-based foods and baby foods for infants and young children, and 1.75 mg/kg for unprocessed durum wheat, oats and maize.[4] In Japan, the contamination level of DON in wheat is provisionally regulated at 1.1 mg/kg.

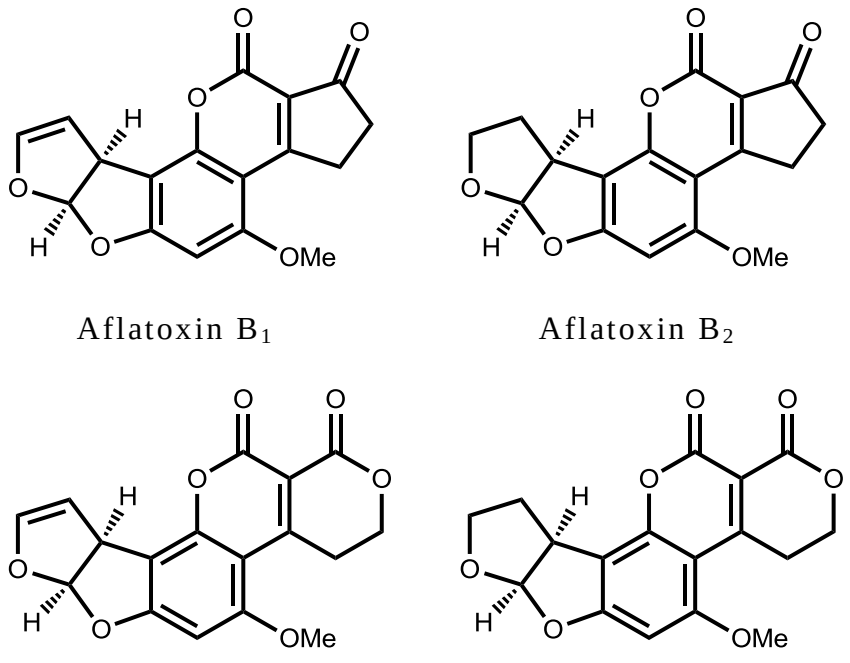
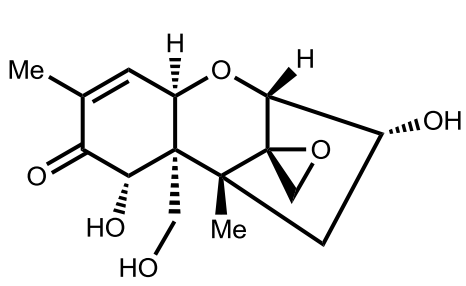


Fig. 1-1. Chemical structures of aflatoxins.



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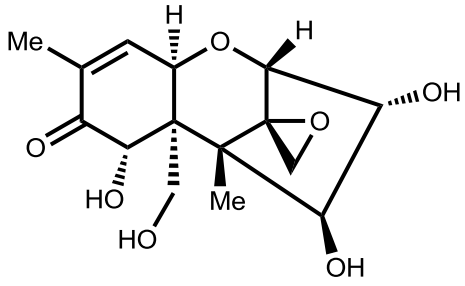
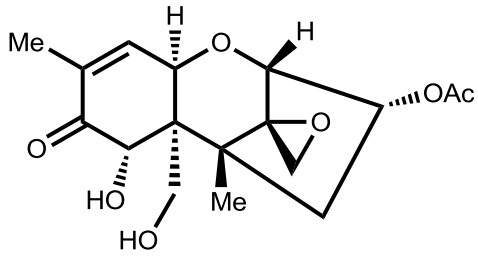
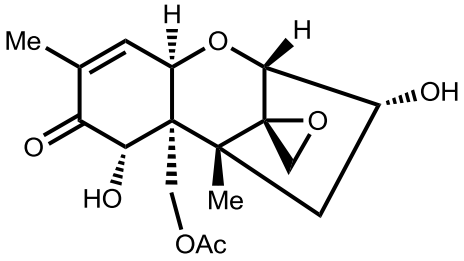


Nivalenol



3-Acetyldeoxynivalenol



15-Acetyldeoxynivalenol

Fig. 1-2. Chemical structures of deoxynivalenol and its analogs.

Ochratoxin A (Fig. 1-3) is a mycotoxin produced by some species of *Aspergillus* and *Penicillium*, and it contaminates various foods, grains, pulse, dry fruits, and beverages.[5,6)] Ochratoxin A has a phenylalanine portion in its structure, and it has shown toxicity for liver and kidney in animal experiments and carcinogenicity for humans. In the EU, regulation values for ochratoxin A contamination are established against individual foods: 0.50 µg/kg for processed cereal-based foods and baby foods for infants and young children, and dietary foods for special medical purposes intended specifically for infants;10 µg/kg for dried vine fruit (currants, raisins and sultanas) and soluble coffee (instant coffee).[7)] In Japan, regulation values have not yet been established for

ochratoxin A contamination against any food.

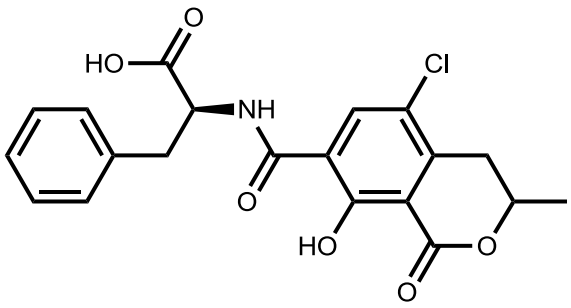


Fig. 1-3. Chemical structure of ochratoxin A.

Patulin, first isolated in 1940 from a culture of *Penicillium patulum* (also called *P. griseofulvum*)[8] is a polyketide mycotoxin and a major contaminant for apples and pears.[9] Patulin shows immunotoxic and neurotoxic effects in animal experiments,[10,11] and is regulated in many countries, including the US, EU, and Japan. These countries have adopted 50 μg/kg as a maximum residue limit in apple juice and apple-related foods. Many species of *Penicillium* and *Aspergillus* such as *P. patulum*, *P. melinii*, *P. claviforme*, *A. clavatus*, *A. giganteus*, and *A. terreus* produce patulin, and the most common species in patulin contamination in apples is *P. expansum*.[9]

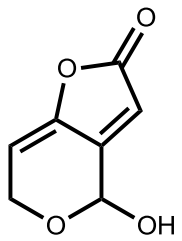


Fig. 1-4. Chemical structure of patulin.

Patulin contamination in foods causes great economic loss, and food industries are eager to develop a technique to prevent patulin contamination in apple juice. Many investigations to prevent or control the growth of *P. expansum* and patulin contamination have been performed, but patulin contamination of apples is still not adequately controlled. Thus, toward the development of new approaches to control patulin contamination in apples, I have studied the effects of plant volatile compounds on patulin production by *P. expansum* in apple juice.

This dissertation consists of four chapters. Chapter 1, this chapter, is an introduction to the present circumstances surrounding the patulin contamination of apples and other foods. Chapter 2 describes the effects of aliphatic aldehydes on the patulin production by *P. expansum* in apple juice, and in particular, the finding that aliphatic aldehydes with 8–10 carbons enhanced patulin production by increasing the patulin biosynthesis gene transcript. In Chapter 3, I illustrate that the composition of volatile compounds in apple juice broths has major effects on the patulin production and growth of *P. expansum*. Chapter 4

explains the conclusions drawn from this research and provides speculation about a new approach to prevent patulin contamination in apples and apple products.

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Chapter 2

Effects of Aliphatic Aldehydes on Fungal Growth and the Patulin Production of *Penicillium expansum* in Apple Juice

2.1 Introduction

Patulin has shown its toxicity, i.e., immunotoxic and neurotoxic effects, in animal experiments.[1,2] In 1995, the Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Joint Expert Committee on Food Additives (JECFA) established the provisional maximal tolerable daily intake of patulin as 0.4 μg/kg of body weight/d.[3] In 2003, the Codex Alimentarius Commission (CAC) set the maximum residue level of patulin at 50 μg/kg for apple juice.[4] Since then, the US, the EU, Japan and many other countries have introduced 50 μg/kg as the maximum patulin residue limit for apple juice and apple-related foods. The patulin contents in apple juice and apple-related foods have been determined in many countries, and some examples of the patulin contamination level were found to exceed the level permitted by the CAC.[5–7]

Since the patulin contamination of foods causes great economic loss, food industries are eager to develop a technique to protect apple juice from patulin contamination, and many investigations to prevent or control the growth of *P. expansum* and patulin contamination have been performed. Since the patulin production in apples usually occurs after

the apples' harvesting, the appropriate quality control of post-harvest apples, such as adequate handling and storage conditions, is very important to prevent patulin contamination.[8–12] The CAC has been preparing guidelines to reduce patulin in apples. Chemical agents such as potassium sorbate and sodium propionate inhibit *P. expansum* growth and patulin production,[8,13] and UV irradiation reduces the amount of patulin in apple cider.[14] However, the patulin contamination of apples is not yet adequately controlled.

It is now widely known that some natural chemicals contributing to plant aroma have antibacterial and antifungal activities.[15–21] In particular, cinnamaldehyde, benzaldehyde, and aliphatic aldehydes have been found to have potent effects against microorganisms. Among these, (*Z*)-3-hexenal, 2,4-hexadienal, and (*E*)-2-undecenal showed relatively strong antibacterial and antifungal effects. (*E*)-2-Hexenal was found to be effective for pears contaminated with *P. expansum*.[22,23] Conversely, gaseous (*E*)-2-heptenal, (*E*)-2-octanal, and (*E*)-2-nonenal have been reported to stimulate aflatoxin production by *A. flavus* inoculated into corn, while suppressing it in cotton seed and peanuts.[24]

In this chapter, to determine the effect of aliphatic aldehydes on fungal growth and patulin production, I applied aliphatic aldehydes with 3–10 carbons, including (*E*)-2-hexenal, to *P. expansum* in apple juice. The aliphatic alkenals with three to six carbons, that is, 2-propenal, (*E*)-2-butenal, (*E*)-2-pentenal, and (*E*)-2-hexenal, inhibited both fungal

growth and spore germination, and the aliphatic aldehydes with eight carbons, octanal and (*E*)-2-octenal, significantly enhanced the patulin production in apple juice. A real time reverse transcription-polymerase chain reaction (RT-PCR) analysis of the biosynthetic genes indicated that the transcription of a patulin biosynthetic gene was activated by aliphatic aldehydes with eight carbons.

2.2 Materials and Methods

2.2.1 Chemicals

Patulin standard was purchased from Wako Pure Chemical Industries (Osaka, Japan). The 16 aliphatic aldehydes and Tween 80 were of reagent grade. EtOH, MeOH, and NaCl were of analytical grade, and MeCN was of HPLC grade.

2.2.2 Culture broths

Apple juice broths were prepared by the sterilization of commercially available apple juice (Meiji, Tokyo, Japan) at 121°C for 10 min. Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from Eiken Chemical (Tochigi, Japan) and BD Difco (Franklin Lakes, NJ, USA) respectively.

2.2.3 Fungal strain and spore preservation

Penicillium expansum ATCC28876 was purchased from the

American Type Culture Collection (Rockville, MD, USA). A physiological saline suspension of mycelia prepared from the culture of *P. expansum* in PDB was inoculated onto a PDA plate, followed by incubation for 7 d or more at 25°C. The spores generated were suspended in 0.05% Tween 80 physiological saline solution and collected in a tube after filtration through a sterile cotton mesh. *P. expansum* spores were preserved to maintain the potential for patulin production by the method reported by Santos et al.[25)]

2.2.4 Preparation of fresh spore suspension

A portion of preserved spores was spread on a PDA plate and cultured for 7 d or more at 25°C. The spores were harvested from the plate and suspended in 0.05% Tween 80 physiological saline solution. After filtration through a cotton mesh, the spores in the suspension were counted under a microscope.

2.2.5 Culture conditions

P. expansum spores were inoculated into apple juice broths (10 mL) containing aliphatic alkenals in 50-mL cell culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA) to a concentration of 1×10^4 spores/mL and cultured statically at 25°C. The aliphatic aldehydes in EtOH were added to the flasks immediately before fungal inoculation, and the concentration of EtOH in the broth was fixed at 0.1%. The broth

containing 0.1% EtOH was used as the control.

2.2.6 Measurement of fungal growth

P. expansum cultures were filtered through No. 2 filter paper (Advantec Toyo Kaisha, Tokyo, Japan) after incubation, and the residue on the paper was collected and put into a tube. The collected mycelia were frozen at −80°C and lyophilized with an FD-81 Freeze Dryer (Tokyo Rikakikai, Tokyo, Japan). The lyophilized mycelia were weighed, and fungal growth was expressed as dry weight.

2.2.7 Determination of the patulin concentration in the apple juice broths

P. expansum cultures in apple juice broths were filtered through No. 2 filter paper. Each filtrate was mixed with an equal volume of MeOH, filtered through a 0.2-µm Ultrafree-MC Centrifugal Filter (Merck Millipore, Billerica, MA, USA), and subjected to a high-performance liquid chromatography (HPLC) analysis with an HP-1100 high-performance liquid chromatograph equipped with a TSK gel ODS-100V separation column (2 × 150 mm, 3 µm; Tosoh, Tokyo, Japan). The HPLC system was connected to a UV detector and a mass selective detector with an electron spray ionization interface (Agilent Technologies, Santa Clara, CA). The mobile phase A was Milli-Q water, and the mobile phase B was MeCN. Separation was done at a flow rate of

0.2 mL/min by the following linear gradient program: from 0.0 to 5.0 min, 95% A, 5% B; from 5.0 to 13.0 min, 95% A, 5% B to 0% A, 100% B; from 13.0 to 15.0 min, 0% A, 100% B; from 15.0 to 15.1 min, 0% A, 100% B to 95% A, 5% B; and from 15.1 to 25.0 min, 95% B, 5% A.

The column temperature was 40°C and the injection volume was 2.0 µL. The UV absorbance at 290 nm and mass spectrometry at *m/z* 153.0 (negative, single-ion monitoring mode) were used in quantitative and qualitative analyses, respectively. The capillary voltage, fragmentor voltage, and temperature for mass spectrometry were –6,000 V, 60 V, and 340°C, respectively. For the calibration standards, patulin standard solutions (0, 6, 26, 45, 65, 260, 450, and 650 µmol/L) were prepared using 5% MeCN. The measured patulin concentration was expressed as the concentration in the apple juice broth.

2.2.8 Fluorescence-staining of fungal mycelia

P. expansum spores were inoculated into apple juice broths and pre-cultured statically at 25°C for 24 h. After confirmation of the germination of spores, 50 µL of 17.8 mmol/L 2-propenal or 80 µL of 119 mmol/L (*E*)-2-pentenal solution in EtOH was added to 10 mL of the fungal culture, which was statically incubated at 25°C. After 2 h and 4 h, fungal mycelia were collected with PTFE membrane filters (Merck Millipore; pore size 5.0 µm), washed twice, and resuspended in physiological saline solution. Twenty µL of Bacstain CFDA

(5(6)-carboxyfluorescein diacetate) solution (Dojindo Laboratories, Kumamoto, Japan) was added to 1 mL of fungal mycelia suspension, and this was mixed gently with a vortex mixer and incubated at 37°C for 15 min. Then 1 µL of Bacstain PI solution (Dojindo Laboratories) was added to 1 mL of fungal mycelia suspension, and this was mixed gently with a vortex mixer and incubated under darkness at atmospheric temperature for 10 min. Stained fungal mycelia were observed by optical microscopy (BX50; Olympus, Tokyo, Japan) and fluorescence microscopy (BX-FLA; Olympus; 6-CFDA: λEx = 493 nm, λEm = 515 nm; PI: λEx = 530 nm, λEm = 620 nm).

2.2.9 Counting of viable spores with growth potential

P. expansum spores were inoculated to a final concentration of 1×10^4 spores/mL into apple juice broths containing 89 µmol/L of 2-propenal or 950 µmol/L of (*E*)-2-pentenal and cultured statically at 25°C. The spores were collected with mixed cellulose ester membrane filters (Merck Millipore; pore size 0.45 µm) from 0.5-, 1-, and 2-d-old cultures, washed three times with 0.05% Tween 80 physiological saline solution, and resuspended in physiological saline solution respectively. Each suspension was adjusted to 1×10^5 spores/mL; 100 µL of the suspension was spread onto PDA plates, and the plates were incubated at 25°C for 7 d. The numbers of colonies that formed on the PDA plates were counted and considered viable spores with growth potential.

2.2.10 Relative expression of genes likely to be involved in patulin biosynthesis

The relative expression of the genes related to patulin biosynthesis in *P. expansum* was evaluated by real-time PCR, as reported by Sanzani et al.[26] The 6-methylsalicylic acid synthase gene (*msas*), the isoepoxydone dehydrogenase gene (*IDH*), and the ATP-binding cassette transporter gene (*peab1*) were selected as target genes for the evaluation of expression, and the β-tubulin (*β-Tub*) gene was selected as a housekeeping gene. Total RNA was extracted from 100 mg of fungal mycelia with ZR Fungal/Bacterial RNA MiniPrep (Zymo Research, Orange, CA, USA) and treated with RNase-free DNase I (Zymo Research). cDNAs were synthesized using a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). Reactions of reverse transcription were carried out using gene-specific primers[26] in a thermal cycler, the GeneAmp PCR System 9700 (Invitrogen Life Technologies, Carlsbad, CA, USA).

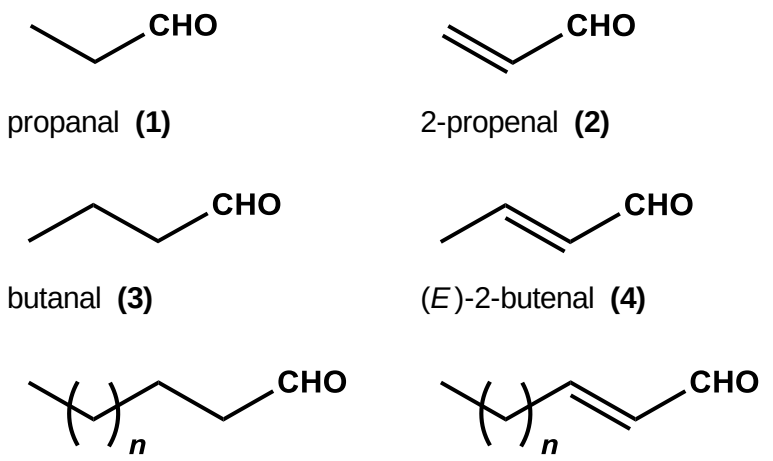
The set of primers used in the cDNA synthesis and SYBR Premix Ex Taq (Takara Bio) were used in a real-time PCR reaction, and PCR amplification was carried out using an ABI PRISM 7300 Real-Time PCR System (Invitrogen Life Technologies). The cycle thresholds of each gene were generated automatically by the software associated with the equipment.

The relative expression of the three target genes was evaluated by the ΔCt (cycle time) method and the ΔΔCt method.[27] Relative expression was calculated by the following formula: $2^{(-\Delta\Delta Ct)}$, where ΔCt = average Ct of a housekeeping gene − average Ct of a target gene, and ΔΔCt = average ΔCt of a fungal culture in the broth containing aliphatic aldehydes – average ΔCt of the control. The resulting data were transformed to $\log_2$, and the levels of change (increase or decrease) were categorized as follows: low ≥ –1.0 to ≤ 1.0; medium ≥ –2.0 to < –1.0, or > 1.0 to ≤ 2.0; high < –2.0, or > 2.0.[28]

2.3 Results

2.3.1 Effects of aliphatic aldehydes on *P. expansum* growth and patulin production

Sixteen aliphatic aldehydes (Fig. 2-1) were added to apple juice broth, and their effects on *P. expansum* growth and patulin production were investigated. The final concentration of aldehydes in the apple juice broth was fixed at the equivalent molar concentration value of 100 μg/mL, and a spore suspension of *P. expansum* was inoculated into the broth to a final concentration of 1×10^4 spores/mL. After incubation at 25°C for 7 d, the dry weight of mycelia was measured as an indicator of fungal growth, and the patulin amount in the culture broth was determined by liquid chromatography/mass spectrometry (LC/MS) (Table 2-1).



propanal **(1)**

2-propenal **(2)**

butanal **(3)**

(*E*)-2-butenal **(4)**

n = 1; pentanal **(5)**

n = 2; hexanal **(7)**

n = 3; heptanal **(9)**

n = 4; octanal **(11)**

n = 5; nonanal **(13)**

n = 6; decanal **(15)**

n = 1; (*E*)-2-pentenal **(6)**

n = 2; (*E*)-2-hexenal **(8)**

n = 3; (*E*)-2-heptenal **(10)**

n = 4; (*E*)-2-octenal **(12)**

n = 5; (*E*)-2-nonenal **(14)**

n = 6; (*E*)-2-decenal **(16)**

Fig. 2-1. Chemical structures of the tested aliphatic aldehydes.

As shown in Table 2-1, 2-propenal (**2**), (*E*)-2-butenal (**4**), (*E*)-2-pentenal (**6**), and (*E*)-2-hexenal (**8**) completely inhibited the growth of *P. expansum*. In accord with this finding, patulin was not detected in the broths. These alkenals have a common structure: six or fewer carbons and an *E* double bond at the α-position, except for 2-propenal, which has no stereoisomers.

Next, the effects of various concentrations of these alkenals on *P. expansum* growth and patulin production were examined. The alkenals inhibited both fungal growth and patulin production in a dose-dependent manner (data not shown). 2-Propenal was the most potent inhibitor. It

Table 2-1 Effects of Aliphatic Aldehydes on *P. expansum* Growth and Patulin Production

Compound	Aldehyde Concentration (mmol/L) [a]	Dry weight of mycelia (mg) [b]	MIC (μmol/L) [c]	Patulin cocentration (mmol/L) [d]
Control	-	43 ± 8	- [e]	0.31 ± 0.14
Propanal **(1)**	1.72	51 ± 11	-	0.07 ± 0.03
2-Propenal **(2)**	1.78	N.D. [f]	89	N.D.
Butanal **(3)**	1.39	39 ± 6	-	0.38 ± 0.07
(*E*)-2-Butenal **(4)**	1.43	N.D.	713	N.D.
Pentanal **(5)**	1.16	33 ± 7	-	0.45 ± 0.09
(*E*)-2-Pentenal **(6)**	1.19	N.D.	950	N.D.
Hexanal **(7)**	1.00	43 ± 4	-	0.09 ± 0.03
(*E*)-2-Hexenal **(8)**	1.02	N.D.	815	N.D.
Heptanal **(9)**	0.88	48 ± 8	-	0.12 ± 0.10
(*E*)-2-Heptenal **(10)**	0.89	42 ± 13	-	0.32 ± 0.14
Octanal **(11)**	0.78	48 ± 8	-	2.49 ± 1.02
(*E*)-2-Octenal **(12)**	0.79	34 ± 16	-	2.46 ± 1.11
Nonanal **(13)**	0.70	52 ± 15	-	1.33 ± 0.60
(*E*)-2-Nonenal **(14)**	0.71	61 ± 19	-	0.66 ± 0.26
Decanal **(15)**	0.64	44 ± 2	-	0.79 ± 0.42
(*E*)-2-Decenal **(16)**	0.65	47 ± 8	-	0.47 ± 0.22

[a]The concentration of aliphatic aldehydes was fixed at the equivalent molar concentration values of 100 μg/mL in the apple juice broth.

[b]Dry weights of mycelia are shown as means ± SD of five independent experiments, and the detection limit was 1 mg.

[c]Minimal inhibitory concentrations were measured only for the compounds that inhibited fungal growth at the equivalent molar concentration values of 100 μg/mL.

[d]Patulin concentrations of the culture broth are shown as means ± SD of five independent experiments, and the detection limit was 12 μmol/L.

completely inhibited fungal growth and patulin production even at 89 μmol/L. (*E*)-2-Butenal, (*E*)-2-pentenal, and (*E*)-2-hexenal also completely inhibited both fungal growth and patulin production, at 713, 950, and 815 μmol/L, respectively.

In contrast, the alkanals and alkenals consisting of 8–10 carbons, that is, octanal (**11**), (*E*)-2-octenal (**12**), nonanal (**13**), (*E*)-2-nonenal (**14**), decanal (**15**), and (*E*)-2-decenal (**16**), significantly enhanced the patulin accumulation ($p < 0.05$, Aspin-Welch T-test). In particular, the patulin concentrations in the culture broth containing octanal and (*E*)-2-octenal were 7.9 and 7.8 times higher, respectively, than that of the control. The eight alkanals consisting of 3–10 carbons and the four alkenals consisting of seven carbons or more did not affect fungal growth at the equivalent molar concentration values of 100 μg/mL.

2.3.2 Effects of alkenals with three and five carbons on the viability of *P. expansum* mycelia and spores

(*E*)-2-Pentenal (**6**) was spiked into a pre-culture of *P. expansum* in apple juice broth (final concentration, 950 μmol/L), and the effect on fungal mycelia was investigated by vital staining. Before the spiking of (*E*)-2-pentenal, almost all of the fungal mycelia were stained green with CFDA, whereas only small parts of mycelia were stained red with PI, indicating that most of the mycelia were alive (Fig. 2-2, panels A-2, A-3). After 2 h of incubation with (*E*)-2-pentenal, green mycelia

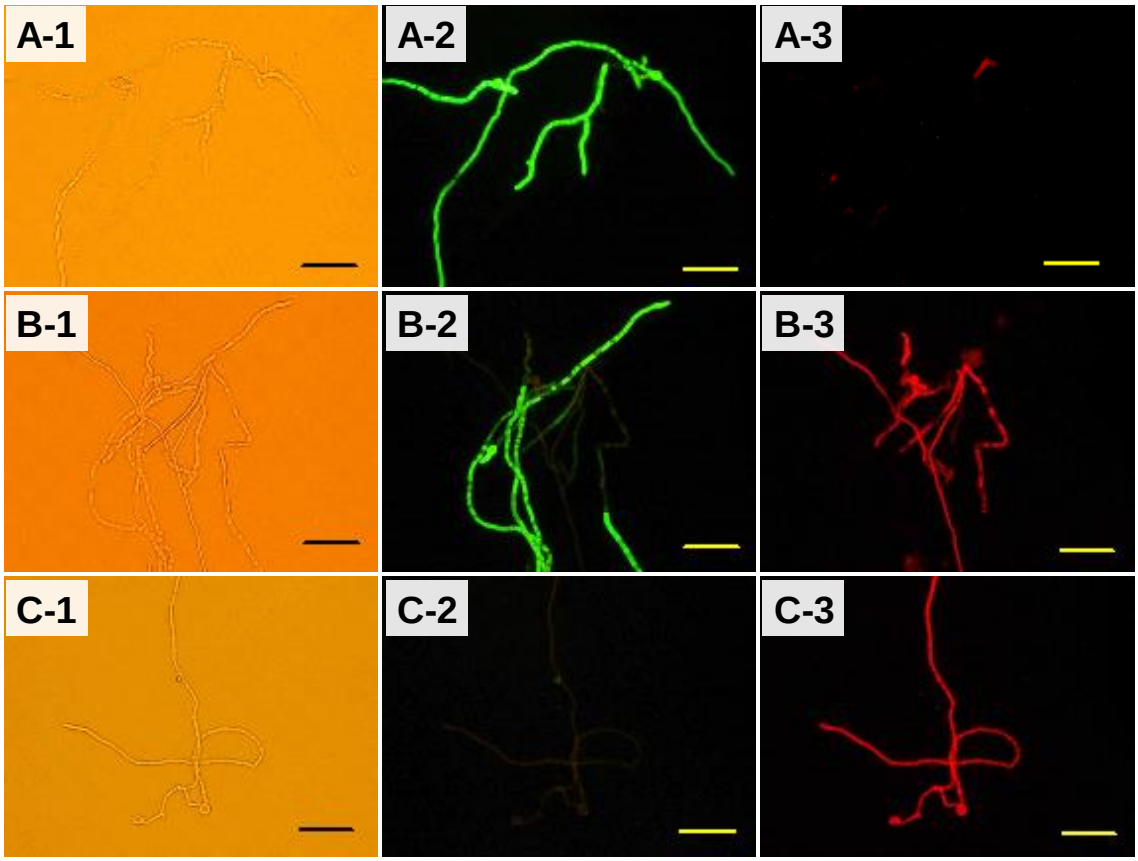


Fig. 2-2. Fluorescence staining of fungal mycelia incubated with (*E*)-2-pentenal.

Mycelia were stained with CFDA and PI, and were microscopically inspected at 0 h (A), 2 h (B), and 4 h (C) after the addition of (*E*)-2-pentenal at 950 µmol/L. Optical microscopic observation (1) and fluorescence microscopic observation with Olympus mirror units U-MWB (2) and U-MWIG (3) were carried out. Bars=50 µm.

decreased and red mycelia increased in number (Fig. 2-2, panels B-2, B-3). After 4 h of incubation, all of the mycelia were stained red, indicating that all had died out (Fig. 2-2, panel C-3). Treatment with 2-propenal (**2**) at a final concentration of 89 µmol/L gave a similar

result.

The effects of aliphatic alkenals with 3–6 carbons on the spores were also examined. Spores were incubated in apple juice broth containing 89 µmol/L of 2-propenal or 950 µmol/L of (*E*)-2-pentenal for appropriate durations, then spread on PDA plates and incubated.

The numbers of colonies that formed on the PDA plates was considered an indicator of spore viability. As shown in Table 2-2, incubation with 2-propenal for 0.5 d suppressed colony formation by 98.6%, and no colonies were formed by spores incubated with 2-propenal for 1 d. Incubation with (*E*)-2-pentenal for 1 d decreased colony formation by 92.0%, and 2 d of incubation completely suppressed colony formation.

In addition, the sizes of the spores incubated in apple juice broth containing these alkenals were measured. In the apple juice broth without alkenals, the mean dia. of the *P. expansum* spores increased from 3.4 µm to 6.9 µm during 12 h of incubation, and the spores germinated on day 1 at a germination rate of 55 ± 4.6%. In contrast, the sizes of the spores did not increase during incubation with 2-propenal at 89 µmol/L or with (*E*)-2-pentenal at 950 µmol/L. Vital staining was attempted for the spores treated with 2-propenal and (*E*)-2-pentenal. The spores without treatment with alkenals were stained by CFDA but not by PI, whereas those treated with alkenals were not stained by either dye. This might have been because PI did not penetrate the spores regardless of

viability.

Table 2-2 Decreases in the Numbers of Viable Spores in Apple Juice Containing 2-Propenal and Containing (*E*)-2-Pentenal

Incubation (d)	Concentration of viable spores (cfu/mL) [a]		
	Control	2-Propenal [b]	(*E*)-2-Pentenal [b]
0	50,667 ± 12,858	-	-
0.5	48,667 ± 8,083	67 ± 14	3,907 ± 959
1	46,000 ± 12,166	< 10 [d]	41 ± 10
2	n. d. [c]	< 10 [d]	< 10 [d]

[a]The numbers of viable spores in the apple juice broth were determined as the concentration of colonies that formed on a PDA plate in colony-forming units (cfu), and is shown as the mean ± SD of three independent experiments.

[b]Spores were treated with 2-propenal at 89 µmol/L and with (*E*)-2-pentenal at 950 µmol/L, spread on PDA plates, and incubated at 25°C.

[c]n.d.: not determined. The colony forming units on day 2 in apple juice broth without alkenals were not measurable due to spore germination.

[d]The detection limit for numbers of viable spores was < 10 cfu/mL.

2.3.3 Effects of aliphatic aldehydes with eight carbons on *P. expansum* growth and patulin production

Spores of *P. expansum* were inoculated into apple juice broth containing octanal (**11**) at concentrations from 78 µmol/L to 7.8 mmol/L and (*E*)-2-octenal (**12**) at concentrations from 79 µmol/L to 7.9 mmol/L.

After incubation at 25°C for 7 d, the dry weight of mycelia and the patulin concentration were determined. As shown in Figure 2-3A, fungal growth was not affected at 78 µmol/L to 2.3 mmol/L of octanal, but was partially inhibited at 7.9 mmol/L. At 79 and 240 µmol/L of (*E*)-2-octenal, fungal growth was not affected, but at 790 µmol/L the mycelia dry weights decreased, and at 2.4 mmol/L the fungal growth was completely inhibited. On the other hand, as shown in Figure 2-3B, the patulin concentration in the culture broth was significantly increased by

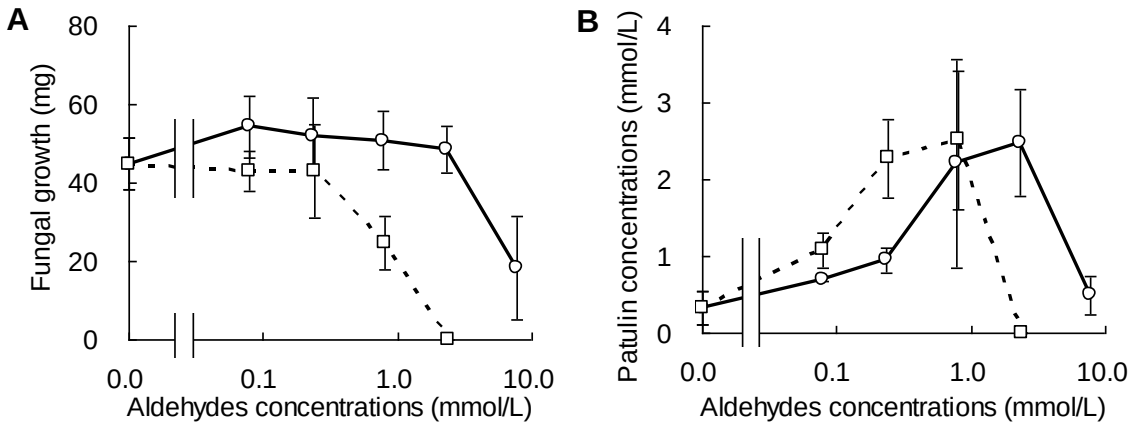


Fig. 2-3. Effects of aliphatic aldehydes with eight carbons on *P. expansum* growth (A) and patulin production (B).

The mycelia dry weights and the patulin concentration in the culture media were determined after the incubation of *P. expansum* in apple juice broth containing from 78 µmol/L to 7.8 mmol/L of octanal (○) or from 79 µmol/L to 7.9 mmol/L of (*E*)-2-octenal (□) at 25°C for 7 d.

Symbols and error bars represent means ± SD respectively of five independent experiments.

treatment with 78 µmol/L to 2.3 mmol/L of octanal and with 79 to 790 µmol/L of (*E*)-2-octenal ($p < 0.05$, Aspin-Welch T-test). The patulin concentrations in the broth containing 2.3 mmol/L of octanal and 790 µmol/L of (*E*)-2-octenal were 8.6 and 7.8 times as high as that in the culture broth without these aldehydes, respectively.

I also monitored the changes in the dry weight of mycelia and the patulin concentration during cultivation at 25°C in apple juice broth containing 2.3 mmol/L of octanal (Fig. 2-4). The dry weight of mycelia in the broth containing octanal was not significantly different from that in the broth without octanal throughout the experimental period (Fig. 2-4A). Figure 2-4B shows the patulin concentrations in the broths incubated with and without 2.3 mmol/L of octanal. At 3.5 d, the patulin concentrations in the two broths were almost the same. However, at both 5 d and at 7 d, the patulin concentrations in the broth with octanal were significantly higher than those without octanal ($p < 0.05$, Aspin-Welch T-test). In addition, from 3.5 d to 5 d, there was a 6.3-fold increase in the patulin concentration in the broth with octanal, whereas only a 1.7-fold increase was observed in that without octanal. From 5 d to 7 d, no significant change in the patulin concentration was observed in either broth. This indicates that stimulation of patulin biosynthesis by octanal occurred to a remarkable extent from 3.5 d to 5 d.

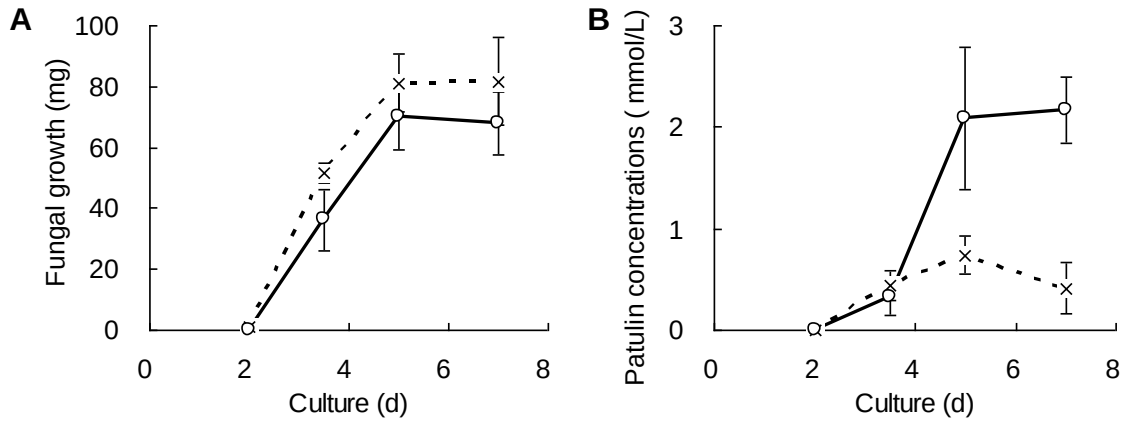


Fig. 2-4. Changes in the dry weight of mycelia (A) and patulin concentrations (B) during *P. expansum* cultivation.

P. expansum spores were inoculated into apple juice broth containing 2.3 mmol/L of octanal (○) or without octanal (×) and incubated at 25°C. The dry weight of mycelia and the patulin concentration were determined 2, 3.5, 5, and 7 d after inoculation. Symbols and error bars represent means ± SD respectively of five independent experiments.

2.3.4 Relative expressions of the genes involved in patulin production and accumulation in the fungal culture with octanal

Three genes, *msas*, *IDH*, and *peab1*, might be involved in patulin production and accumulation: *msas* encodes 6-methylsalicylic acid (6-MSA) synthase, which catalyzes the formation of 6-MSA from acetyl-CoA and malonyl-CoA as the first step in the patulin biosynthetic pathway; *IDH* encodes isoepoxydone dehydrogenase, which transforms isoepoxydone to phyllostine; and *peab1* encodes the ATP-binding cassette (ABC) putative transporter for patulin efflux. These three genes

have been reported to be upregulated during patulin biosynthesis.[28] The Cts of the three genes were measured by the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) method, and the ΔCt value for each gene was estimated using the Ct value of *β-Tub* as a housekeeping gene. Next, the ΔΔCt value for each gene was calculated by subtracting the ΔCt value of the fungal culture in the broth containing octanal from that for the control. The relative expression of each gene is expressed as the $\log_2$ (–ΔΔCt) value (Fig. 2-5).

As shown in Figure 2-5, the $\log_2$ (–ΔΔCt) values for the *msas* gene in the 3.5- and the 5-d-old cultures were 2.2 and 2.3, respectively. This indicates that the addition of octanal significantly enhanced the transcription of the *msas* gene ($p < 0.05$, Aspin-Welch T-test), whereas no significant transcription activation of the other two genes was detected. In the 5-d-old culture, the $\log_2$ (–ΔΔCt) value for the *msas* gene was 2.3, but the amounts of the other two genes' transcripts did not increase, indicating that the addition of octanal activated the transcription of the *msas* gene in both the 3.5- and the 5-d-old cultures, whereas the transcription of the other two genes was not affected by octanal. In the 7-d-old culture, the $\log_2$ (–ΔΔCt) values for the *IDH*, *msas*, and *peab1* genes were –0.5, 0.6, and –0.4, respectively, indicating that in the 7-d-old culture, octanal no longer affected the transcription of these three genes.

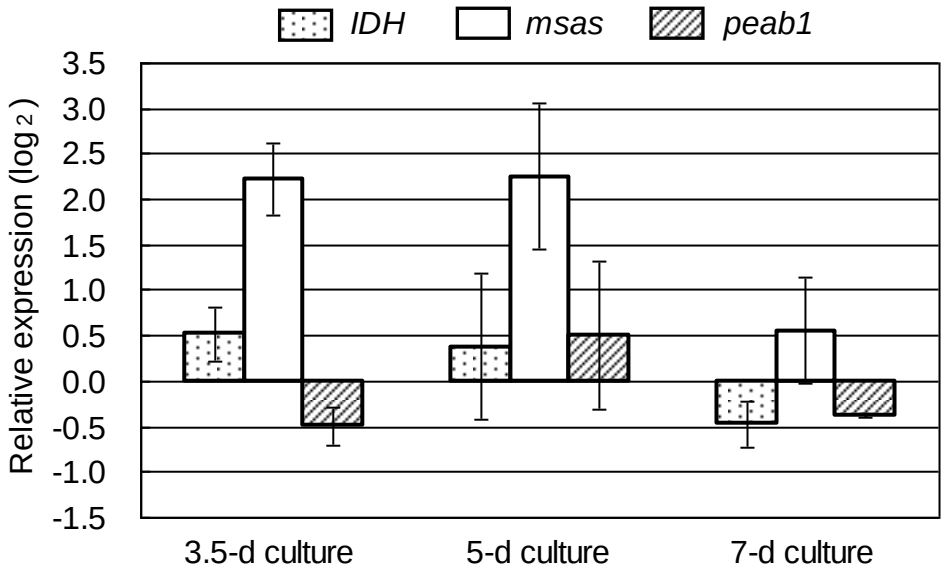


Fig. 2-5. Relative expression of the genes probably involved in the patulin biosynthesis of *P. expansum* cultured in apple juice broth containing octanal.

The relative expression of the *IDH*, the *msas*, and the *peab1* genes in *P. expansum* grown for 3.5, 5, and 7 d at 25°C in apple juice broth containing 2.3 mmol/L of octanal to control are shown. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method, and were normalized using the *β-Tub* gene as the housekeeping gene. Data and error bars represent means ± SD respectively of three independent experiments.

2.4 Discussion

The results of the present study indicate that the effects of aliphatic aldehydes on *P. expansum* growth and patulin production depend on the carbon chain length and the presence of a double bond. Aliphatic aldehydes consisting of six carbons or less and having a double

bond at the α-position were cytotoxic, whereas those consisting with eight carbons or more stimulated patulin production.

The effects of the 16 aliphatic aldehydes with 3–10 carbons on the growth and patulin production of *P. expansum* were examined using apple juice broth and *P. expansum* spores. The results revealed that 2-propenal (**2**), (*E*)-2-butenal (**4**), (*E*)-2-pentenal (**6**), and (*E*)-2-hexenal (**8**) had inhibitory effects against *P. expansum* growth. The minimum inhibitory concentrations (MICs) were 89, 713, 950, and 815 μmol/L, respectively. The vital staining of the mycelia clearly indicated that these alkenals caused a loss of viability in the mycelia. Treatment by these alkenals also caused a loss of germination potential in the fungal spores.

In a study by Kubo et al. of the antifungal activity of a series of aliphatic aldehydes with 5–14 carbons against *Saccharomyces cerevisiae*, alkanal and alkenal with 11 carbons showed the strongest antifungal activity.[21)] These aliphatic aldehydes were composed of hydrophobic and hydrophilic regions, and thus they were able to act as nonionic surfactants. On the basis of the structure-activity relationship, Kubo et al. suggested that their ability to act as surfactants is important to the expression of antifungal activity. However, in the present experiments, the smallest alkenal, 2-propenal, showed the strongest activity, and the corresponding alkanals were not effective. Thus the mode of action of the antifungal activity of alkenals with 3–6 carbons against *P. expansum*

appears to be different from that proposed for the antifungal activity of aldehydes against *S. cerevisiae*.

The other proposed mechanism of the toxicity of alkenals is an interaction with molecules with nucleophilic groups, including glutathione (GSH), and biomacromolecules such as proteins[29,30] and DNA.[31,32] There is evidence that alkenals form adducts with these molecules. These adducts are likely to be involved in the biotoxicity of the alkenals, because adduct formation can lead to a shortage of biomacromolecules or a loss of correct function on the part of proteins.[33]

Alkenals bind to the sulfhydryl groups of cysteine residues of GSH and proteins by Michael addition, and to the amino groups of lysine residues and N-terminals of proteins by Schiff-base formation. The structure-activity relationship for the antifungal activity of aldehydes against *P. expansum* was similar to that of the activity for the suppression of intracellular GSH in rat lung cells. In one study, 2-propenal showed the strongest activity for the suppression of GSH, followed by (*E*)-2-butenal and the larger 2-alkenals, whereas the alkanals showed much lower activity.[34] The activity of alkenals for the suppression of GSH was correlated with the rate constants (k_1) for the forward reaction of GSH and alkenals to adducts. Accordingly, it is likely that the formation of adducts by 2-alkenals with nucleophilic groups of biomolecules is involved in the antifungal activity of *P.*

expansum.

In the present study, I found that the treatment of *P. expansum* with aliphatic aldehydes with 8–10 carbons induced a significant increase in the amount of patulin accumulating in apple juice broth without affecting the fungal growth. Octanal was the most effective in enhancing patulin accumulation when aldehydes were applied at 780 μmol/L, inducing a 7.9-fold increase in patulin. Zeringue investigated the effect of the application of gaseous (*E*)-2-heptenal, (*E*)-2-octenal, and (*E*)-2-nonenal on the production of aflatoxin B_1 by *Aspergillus flavus* inoculated into corn, cottonseed, and peanuts.[24)]

Overall, the results of his study showed that increased concentrations of the applied alkenals decreased the amounts of aflatoxin B_1 produced. However, the author reported a significant stimulation of aflatoxin production in *A. flavus*-infected corn culture by the lowest amounts of (*E*)-2-heptenal, (*E*)-2-octenal, and (*E*)-2-nonenal applied. Aflatoxin production was stimulated only when *A. flavus* was inoculated into the corn, and the rate of increase was at most 1.5-fold. It would be interesting to explore whether a common mechanism underlies the phenomena observed in that study and those revealed by the present study, since such an investigation might provide insight into the regulation of mycotoxin production.

The present findings also indicate that the addition of octanal enhanced the transcription of the *msas* gene. An increased amount of

6-MSA synthase in the fungus should lead to the enhanced production of 6-MSA, and this might be one of the mechanisms underlying the stimulation of patulin production and accumulation by octanal. Transcription of the *IDH* and the *peab1* gene was, however, largely unaffected during culturing, suggesting that the steps at which the products of these two genes worked were not the rate-determining steps of patulin accumulation.

6-MSA synthase belongs to the polyketide synthase family, whose members play a role in the biosynthesis of other mycotoxins, e.g., aflatoxins, fumonisins, and ochratoxin. The formation of 6-MSA is the first intermediate in the patulin biosynthetic pathway. It has been reported that an absence of the *msas* and *IDH* genes in the patulin-producing fungus *Byssochlamys fulva* resulted in an inability to produce patulin.[35] In addition, *P. expansum* mutants that show reduced transcription of *msas* produced reduced amounts of patulin.[36] The present results strongly suggest that the step catalyzed by the 6-MSA synthase might be one of the rate-determining steps in patulin biosynthesis, and might prove to be an effective target in efforts to control patulin production.

Here, the transcription of the *msas* gene was enhanced by octanal in the 3.5-d-old cultures, and there was a significant increase in the patulin concentration from 3.5 d to 5 d. The amounts of *msas* transcript in the 5-d-old cultures were similar to those in the 3.5-d-old cultures,

but the patulin concentration did not change from 5 d to 7 d. This difference suggests that the degradation of patulin and/or the depletion of the substrate of 6-MSA synthase starts at about 5 d.

In summary, the results of the present study indicate that some aldehydes have the ability to stimulate patulin biosynthesis in *P. expansum*. The elucidation of the mechanism(s) underlying the stimulation of patulin biosynthesis by aldehydes might give rise to a new approach to the prevention of patulin contamination in apples and other fruits by controlling patulin production in *P. expansum*.

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Chapter 3

Effects of Volatile Compounds in Apple Juices on Fungal Growth and the Patulin Production by *Penicillium expansum*

3.1 Introduction

As described in Chapter 2, some aliphatic aldehydes show antifungal activity against *P. expansum*: 2-propenal, (*E*)-2-butenal, (*E*)-2-pentenal, and (*E*)-2-hexenal added to apple juice broths inhibited the germination of *P. expansum* spores and fungal growth. In contrast, the aliphatic aldehydes with 8–10 carbons, i.e., octanal, (*E*)-2-octenal, nonanal, (*E*)-2-nonenal, decanal, and (*E*)-2-decenal added to the broth significantly enhanced the patulin production in *P. expansum* without affecting fungal growth. These findings suggest that the volatile components of apples and apple juices also affect the patulin production by *P. expansum* growing in apples and apple juices.

Karlshøj et al. (2007) investigated the composition of volatile compounds by electric nose analysis to clarify the relationship between *P. expansum* infection and patulin spoilage in apples.[1)] To the best of my knowledge, however, there is no report on the effects of individual volatile components of apples on the patulin production by *P. expansum*. In this chapter the effects of volatile components of apple juices on the patulin production by *P. expansum* are described, along with the finding that some of the components inhibited fungal growth and others, such as

2-methylbutanoic acid and its ethyl ester, increased the patulin contents concentration-dependently.

3.2 Materials and Methods

3.2.1 Apple juices

Apple juices “A” to “F” made by six different domestic companies in Japan were purchased from grocery stores and stored at 4°C under darkness until the experiments. The from-concentrate apple juices were prepared as follows: apple juices were concentrated by a Rotary Vacuum Evaporator (N-N series) equipped with a Vacuum Controller NVC-1100 (Tokyo Rikakikai Co., Tokyo, Japan) at 45°C, and the concentrates were filled up to their original volumes by adding Milli-Q water. The apple juices thus prepared were also stored at 4°C under darkness until the experiments.

3.2.2 Chemicals

Patulin standard was purchased from Wako Pure Chemical Industries (Osaka, Japan). The 13 volatile compounds were purchased from Wako or from Tokyo Chemical Industry Co. (Tokyo, Japan). EtOH, MeOH, HCl, and NaCl were of analytical grade, and MeCN was of HPLC grade.

3.2.3 Fungal strain and spore preparation

Penicillium expansum ATCC28876 was purchased from the American Type Culture Collection (Rockville, MD, USA). *P. expansum* spore suspension was prepared and preserved as described in Chapter 2.

3.2.4 Culture conditions

P. expansum spores were inoculated to apple juice broths (10 mL) containing volatile compounds in 50-mL cell culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA) with the final spore concentration 1×10^4 spores/mL and cultured statically at 25°C. The volatile compounds in EtOH were added to the flasks immediately before the inoculation. The concentration of EtOH in the broth was 0.1%, and *P. expansum* culture in apple juice broth containing 0.1% EtOH was used as the control.

3.2.5 Measurement of fungal growth

P. expansum cultures in the apple juice broths were filtered through No. 2 filter paper (Advantec Toyo Kaisha, Tokyo, Japan). Collected mycelia were frozen at −80°C and lyophilized. The lyophilized mycelia were weighed, and the fungal growth is expressed as a dry weight.

3.2.6 Determination of patulin concentration in apple juice broth

P. expansum cultures in apple juice broths were filtered through No. 2 filter paper. Each filtrate was mixed with an equal volume of MeOH and then filtered through a 0.2-µm Ultrafree-MC Centrifugal Filter (Merck Millipore, Billerica, MA, USA). The patulin concentration in each filtrate was determined as described in Chapter 2.

3.2.7 Analysis of volatile compounds of apple juices by GC-MS coupled with headspace SPME

A 10-mL aliquot of 1% apple juice in saturated NaCl solution was poured into a 20-mL glass vial, and then 50 µL of 1 mol/L HCl was added. After the addition of 10 µL of a mixture of methyl isobutyl ketone (10 µg/mL) and cyclooctyl alcohol (10 µg/mL) in EtOH as internal standards, the glass vial was sealed with a PTFE-lined cap (GL Sciences Inc., Tokyo, Japan). Volatile compounds in headspace from the apple juice were extracted using a solid phase micro-extraction (SPME) fiber: 50/30 µm DVB/CAR/PDMS, 2-cm length (Sigma-Aldrich, St. Louis, MO, USA). Extraction was performed at 60°C for 40 min under agitation (speed 400 rpm).

After extraction, the SPME fiber was immediately inserted into the GC injector for thermal desorption at 250°C for 5 min. An Agilent 6890 N gas chromatography system coupled to a 5975 inert mass selective detector (MSD; Agilent Technologies, Santa Clara, CA) was used for

capillary GC-MS analyses in the electron impact mode. Volatile compounds were separated using a polyethylene glycol column, DB-WAX (Agilent, 30 m × 0.25 mm, film thickness 0.25 µm).

The chromatographic conditions were as follows: injection system, splitless; injector temperature, 250°C; temperature program, from 0 to 5 min at 40°C, from 5 to 19 min at 15°C /min and from 19 to 29 min at 250°C; carrier gas, He; constant flow, 1.0 mL/min; transfer line to MSD, 250°C. The identification of compounds was made by the comparison of the mass spectra with those in the NIST mass spectra libraries (NIST, Gaithersburg, MD, USA). Linear Kovats indices of authentic compounds were also used to confirm the identification.

3.3 Results

3.3.1 Patulin production by *P. expansum* in six broths made from different commercial apple juices and the effect of the evaporation of apple juices on patulin production

Six apple juice broths were prepared from commercial apple juices A–F made by six different domestic companies by sterilization at 110°C for 10 min. In addition, the apple juices were concentrated by evaporation to partially remove volatile compounds from them, and the evaporated apple juices were filled up to the original volumes by adding water. From these apple juices, apple juice broths were also prepared by sterilization.

The original apple juice broths A–F are referred to herein as Originals A–F, and the apple juice broths prepared from the concentrates are referred to as Evaporateds A–F. A spore suspension of *P. expansum* was inoculated into these broths and incubated at 25°C for 7 d. The dry weight of mycelia was measured as an indicator of fungal growth, and the patulin amount in the culture broth was determined by LC/MS.

Figure 3-1A shows the patulin production by *P. expansum* grown on the six Originals A to F. The difference in apple juices markedly affected the patulin production by *P. expansum*. The lowest concentration of patulin was 0.15 mmol/L in Original E, and the highest patulin concentration was 1.09 mmol/L in Original C. Figure 3-1A also shows the effect of the evaporation of apple juices on the patulin production by *P. expansum* in apple juice broth. The evaporation significantly reduced the patulin concentrations, by 57%, 39%, 54% and 69% in apple juices A, B, C, and E, respectively ($p < 0.01$, Aspin-Welch T-test). In apple juices D and F, the evaporation scarcely changed the patulin concentrations in culture. These results suggested that some volatile compounds of apple juices might stimulate the patulin production by *P. expansum*.

Figure 3-1B shows the fungal growth on the six Original broths. The smallest dry weight of mycelia was obtained in Original D (81% of the average value of the six broths), and the largest dry weight was obtained in Original C (119% of the average value), indicating that

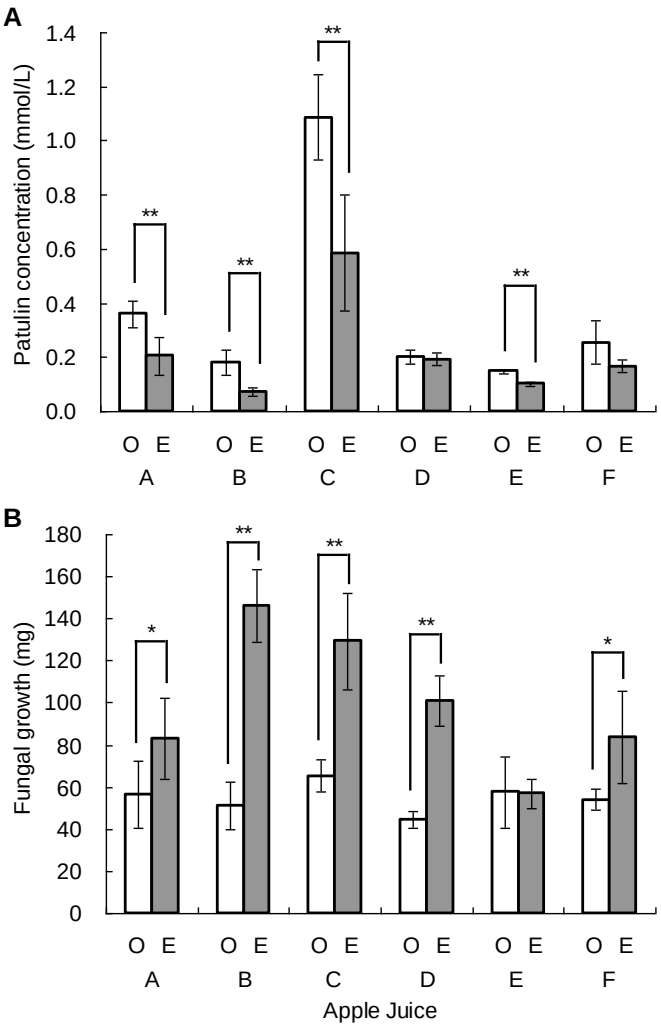

Fig. 3-1. Patulin production (A) and fungal growth (B) in 12 types of apple juice broths.

The patulin concentration (A) and fungal growth (B) in culture media were determined as described in the text after the incubation of *P. expansum* in the apple juice broths at 25°C for 7 d. The blank columns (O) indicate the values for the original juice broths (Originals), and the filled columns (E) are the values for the broths prepared from the concentrates obtained by the evaporation of original apple juices (Evaporateds). The differences in the patulin concentrations and in the dry weights of mycelia were analyzed by t-test. *p<0.05; **p<0.01. Columns and error bars represent the means and SD respectively of five individual experiments.

fungal growth was not greatly affected by the difference in apple juices. Figure 3-1B also shows the fungal growth on the Evaporateds A to F. Except for apple juice E, the evaporation significantly increased the dry weight of mycelia. In particular, in apple juices B, C, and D, the evaporation increased the dry weight of mycelia by 2.9-, 2.0-, and 2.3-fold, respectively. These results suggested that some volatile compounds of apple juices inhibited *P. expansum* growth.

3.3.2 Analysis of volatile compounds in original and evaporated apple juices

Since the volatile compounds in the apple juice might affect the growth of *P. expansum* and its patulin production, the contents of volatile compounds were determined in the original six apple juices A–F and in the six from-concentrate apple juices A–F corresponding to the original ones. The volatile compounds of the original apple juices were analyzed by GC-MS coupled with headspace-SPME, and a total of 57 compounds were detected.

Table 3-1 demonstrates the contents of the 57 compounds in the six original and the six from-concentrate apple juices. All or some of the following 20 compounds were detected in the six original apple juices A–F, but all of them were below the limit of detection (50,000 counts for peak areas) in the six from-concentrate apple juices A–F: *n*-propyl acetate (**1**), 2-methylpropyl acetate (**2**), ethyl butyrate (**3**), ethyl

Table 3-1. Relative Peak Area of Volatile Compounds from Six Apple Juices Determined by GC/MS Analysis Coupled with Headspace SPME

Compound	Detected Fragment; m/z	RT	Apple Juice A [a]		Apple Juice B		Apple Juice C		Apple Juice D		Apple Juice E		Apple Juice F	
			Original [b]	Evaporated [c]	Original	Evaporated	Original	Evaporated	Original	Evaporated	Original	Evaporated	Original	Evaporated
n-Propyl acetate (**1**)	43	4.18	N.D. [d]	N.D.	N.D.	N.D.	N.D.	N.D.	0.167	N.D.	N.D.	N.D.	0.208	N.D.
2-Methylpropyl acetate (**2**)	43	5.18	5.409	N.D.	N.D.	N.D.	1.303	N.D.	2.086	N.D.	2.071	N.D.	0.101	N.D.
Ethyl butyrate (**3**)	71	5.77	1.045	N.D.	12.216	N.D.	0.839	N.D.	7.007	N.D.	2.836	N.D.	0.674	N.D.
Ethyl 2-methylbutanoate (**4**)	57	6.16	2.255	N.D.	0.083	N.D.	2.959	N.D.	3.775	N.D.	0.850	N.D.	0.197	N.D.
Ethyl 3-methylbutanoate (**5**)	88	6.49	N.D.	N.D.	1.840	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Butyl acetate (**6**)	43	6.59	80.532	0.023	2.488	0.022	36.835	0.023	38.300	0.034	23.723	0.025	3.092	0.025
Hexanal (**7**)	44	6.69	0.554	0.039	5.510	0.257	0.104	0.011	2.682	0.115	1.230	0.142	0.122	0.035
2-Methylbutyl acetate (**8**)	43	7.43	14.621	0.031	15.144	0.017	18.315	0.013	9.568	N.D.	2.655	0.013	2.094	0.015
Propyl 2-methylbutanoate (**9**)	57	7.68	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.016	N.D.
Hexyl propanoate (**10**)	57	7.73	0.254	N.D.	0.486	N.D.	0.033	N.D.	N.D.	N.D.	N.D.	N.D.	0.050	N.D.
Pentyl acetate (**11**)	43	8.20	0.035	N.D.	N.D.	N.D.	N.D.	N.D.	0.028	N.D.	N.D.	N.D.	0.062	N.D.
1-Butanol (**12**)	56	8.24	0.818	0.011	0.192	0.007	0.117	N.D.	N.D.	N.D.	N.D.	N.D.	0.162	N.D.
Methyl hexanoate (**13**)	74	8.37	0.063	0.006	0.183	0.012	N.D.	N.D.	0.033	N.D.	0.015	0.008	0.012	0.011
Butyl butyrate (**14**)	71	8.75	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.022	N.D.
2-(*E*)-Hexenal (**15**)	41	8.89	1.247	0.015	2.914	0.077	1.054	0.005	0.526	0.022	0.055	0.021	0.070	0.011
Ethyl hexanoate (**16**)	88	8.94	N.D.	N.D.	0.227	N.D.	0.019	N.D.	0.090	N.D.	N.D.	N.D.	0.053	N.D.
3-Methyl-1-butanol (**17**)	55	9.04	N.D.	N.D.	N.D.	N.D.	0.227	N.D.	0.293	N.D.	0.817	N.D.	N.D.	N.D.
2-Methy-1-butanol (**18**)	41	9.14	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.059	N.D.	0.151	N.D.	0.437	N.D.
Hexyl acetate (**19**)	43	9.49	58.172	0.018	1.418	0.020	26.245	0.038	30.445	0.024	17.554	0.032	1.369	0.029
3-Methylbutyl pentanoate (**20**)	70	9.64	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.162	N.D.	0.144	N.D.	N.D.	N.D.
1-Pentanol (**21**)	42	9.70	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.041	N.D.
Octanal (**22**)	43	9.87	0.023	0.011	0.040	0.011	0.115	0.020	N.D.	0.011	N.D.	0.028	0.032	0.025
(*Z*)-3-Hexenyl acetate (**23**)	67	10.17	N.D.	N.D.	0.223	N.D.	0.036	N.D.	N.D.	N.D.	0.033	N.D.	N.D.	N.D.
(*E*)-2-Hexenyl acetate (**24**)	43	10.37	N.D.	N.D.	4.998	N.D.	N.D.	N.D.	1.042	N.D.	N.D.	N.D.	N.D.	N.D.
1-Hexanol (**25**)	56	10.68	26.016	0.027	12.673	0.025	2.328	0.022	11.025	0.025	5.150	0.031	2.737	0.038
Methyl octanoate (**26**)	74	10.99	0.037	0.012	N.D.	0.022	0.038	0.024	N.D.	0.027	0.038	0.016	0.040	0.023

Table 3-1. Continued

Compound	Detected Fragment; m/z	RT	Apple Juice A [a]		Apple Juice B		Apple Juice C		Apple Juice D		Apple Juice E		Apple Juice F	
			Original [b]	Evaporated [c]	Original	Evaporated	Original	Evaporated	Original	Evaporated	Original	Evaporated	Original	Evaporated
Nonanal (**27**)	57	11.04	0.094	0.054	N.D.	0.055	0.111	0.041	0.127	0.047	0.059	0.042	0.068	0.052
(*Z*)-3-Hexen-1-ol (**28**)	67	11.07	N.D. [d]	N.D.	1.585	N.D.	N.D.	N.D.	0.677	N.D.	0.574	N.D.	N.D.	N.D.
Hexyl 2,2-dimethylpropanoate (**29**)	57	11.22	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.095	N.D.
(*E*)-2-Hexen-1-ol (**30**)	67	11.24	N.D.	N.D.	0.307	N.D.	0.052	N.D.	0.111	N.D.	0.044	N.D.	0.019	N.D.
Acetic acid (**31**)	43	11.58	0.039	0.036	0.041	0.022	0.039	0.027	0.059	0.034	0.035	0.026	0.038	0.023
Furfural (**32**)	96	11.71	0.123	0.027	0.076	0.021	0.028	0.010	0.026	0.014	0.020	0.017	0.007	0.007
2-Ethyl-1-hexanol (**33**)	57	11.95	0.184	0.245	0.214	0.232	0.265	0.246	0.257	0.244	0.243	0.198	0.247	0.167
Methy nonanoate (**34**)	74	11.99	0.065	N.D.	0.063	N.D.	0.071	0.037	0.078	0.036	0.051	N.D.	0.067	0.039
Decanal (**35**)	57	12.07	0.061	0.057	0.066	0.039	0.011	N.D.	0.019	N.D.	N.D.	0.042	N.D.	N.D.
Benzaldehyde (**36**)	106	12.31	0.111	0.031	0.146	0.034	0.047	0.033	0.046	0.033	0.052	0.030	0.145	0.039
Methyl decanoate (**37**)	74	12.90	0.036	N.D.	0.030	0.015	0.035	0.020	0.036	0.016	0.022	0.014	0.027	0.016
Menthol (**38**)	71	13.33	0.017	0.021	0.021	0.017	N.D.	0.017	0.014	0.017	0.017	0.016	0.018	0.014
Acetophenone (**39**)	105	13.42	0.048	0.045	0.047	0.043	0.048	0.044	0.049	0.043	0.047	0.040	0.047	0.039
2-Methyl butanoic acid (**40**)	74	13.49	0.010	0.008	0.009	0.006	0.328	0.034	0.075	0.008	0.009	0.003	0.022	0.002
1-Phenylethyl acetate (**41**)	104	13.73	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.182	N.D.	N.D.	N.D.	N.D.	N.D.
Dimethylglutarate (**42**)	59	13.76	0.042	0.040	0.042	0.043	0.040	0.046	0.050	0.050	0.047	0.034	0.049	0.032
Methy dodecanoate (**43**)	74	14.56	0.062	0.011	0.045	0.019	0.076	0.026	0.045	0.017	0.022	0.019	0.029	0.023
(*E*)-β-Damascenone (**44**)	69	14.78	0.171	0.124	0.145	0.107	0.150	0.112	0.200	0.165	0.117	0.099	0.305	0.247
Hexanoic acid (**45**)	60	14.81	0.034	0.083	0.225	0.096	0.078	0.047	0.182	0.057	0.093	0.031	0.055	0.030
2-Ethyl-3-hydroxyhexyl 2-methylpropanoate (**46**)	71	15.06	1.858	1.916	1.871	1.835	1.778	1.568	1.412	1.394	1.781	1.656	1.640	1.491
Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester (**47**)	71	15.14	0.892	0.694	0.855	0.830	0.837	0.813	1.064	0.845	0.909	0.600	0.768	0.704
2,2-Dimethyl-1-(2-hydroxy-1-methylethyl)propyl 2-methylpropanoate (**48**)	71	15.23	1.317	1.347	1.407	1.391	1.291	1.291	1.274	1.191	1.478	1.157	1.424	1.026

Table 3-1. Continued

Compound	Detected Fragment; m/z	RT	Apple Juice A [a]		Apple Juice B		Apple Juice C		Apple Juice D		Apple Juice E		Apple Juice F	
			Original [b]	Evaporated [c]	Original	Evaporated	Original	Evaporated	Original	Evaporated	Original	Evaporated	Original	Evaporated
α-Calacorene (**49**)	157	15.49	0.286	0.269	0.288	0.282	0.186	0.145	0.184	0.184	0.138	0.114	0.085	0.080
2-Ethyl hexanoate (**50**)	73	15.55	0.243	0.315	0.279	0.359	0.228	0.236	0.267	0.249	0.342	0.188	0.256	0.165
Octanoic Acid (**51**)	60	16.29	0.322	0.334	0.331	0.397	0.286	0.321	0.408	0.380	0.438	0.219	0.310	0.259
Elemol (**52**)	59	16.54	0.127	0.146	0.132	0.132	0.137	0.113	0.099	0.110	0.122	0.103	0.109	0.097
Nonanoic acid (**53**)	60	16.98	0.892	0.694	0.855	0.830	0.837	0.813	1.064	0.845	0.909	0.600	0.768	0.704
Methyl hexadecanoate (**54**)	74	17.37	0.090	0.015	0.060	0.026	0.150	0.043	0.041	0.030	0.032	0.039	0.059	0.041
n-Decanoic acid (**55**)	73	17.64	0.471	0.277	0.407	0.351	0.572	0.374	0.475	0.343	0.371	0.262	0.319	0.284
Methyl octadecanoate (**56**)	74	18.60	0.020	0.006	0.016	0.009	0.040	0.010	0.010	0.008	0.009	0.013	0.011	0.009
Dodecanoic acid (**57**)	73	18.87	0.859	0.352	0.653	0.437	1.170	0.589	0.537	0.478	0.428	0.455	0.367	0.317

[a] Values are relative areas and were calculated as follows: (area counts of a volatile compound) / (area counts of an internal standard). Methyl isobutyl ketone was used as an internal standard for the compounds from n-propyl acetate (**1**) to 1-hexanol (**25**), and cyclooctyl alcohol was used for the compounds from methyl octanoate (**26**) to dodecanoic acid (**57**).

[b] Original; original apple juice.

[c] Evaporated; apple juice from concentrate by evaporation.

[b] N.D., not detected.

2-methylbutanoate (**4**), ethyl 3-methylbutanoate (**5**), propyl 2-methylbutanoate (9), hexyl propanoate (**10**), pentyl acetate (**11**), butyl butyrate (**14**), ethyl hexanoate (**16**), 3-methyl-1-butanol (**17**), 2-methy-1-butanol (**18**), 3-methylbutyl pentanoate (**20**), 1-pentanol (**21**), (*Z*)-3-hexenyl acetate (**23**), (*E*)-2-hexenyl acetate (**24**), (*Z*)-3-hexen-1-ol (**28**), hexyl 2,2-dimethylpropanoate (**29**), (*E*)-2-hexen-1-ol (**30**), and 1-phenylethyl acetate (**41**). This indicated that evaporation reduced the contents of these compounds with relatively low boiling points and short retention times. By contrast, for the relatively high boiling point compounds with long retention times, the detected intensities were hardly affected by evaporation, except for a few compounds.

Thirteen compounds were chosen on the basis of two criteria: (1) the compounds had 0.1 or more of their relative intensity values against internal standards in the original apple juice C, in which the largest amount of patulin was produced among the six original apple juices; and (2) the concentration of the compounds were reduced to 1/5 or less by evaporation. Those compounds were 2-methylpropyl acetate (**2**), ethyl butyrate (**3**), ethyl 2-methylbutanoate (**4**), butyl acetate (**6**), hexanal (**7**), 2-methylbutyl acetate (**8**), 1-butanol (**12**), (*E*)-2-hexenal (**15**), 3-methyl-1-butanol (**17**), hexyl acetate (**19**), octanal (**22**), 1-hexanol (**25**), and 2-methylbutanoic acid (**40**). Their actual concentrations in the original apple juices and the from-concentrate apple juices are measured more accurately by calibration curves obtained with the authentic

Table 3-2. Concentrations of Thirteen Volatile Compounds of Six Commercial Apple Juices

Compound	Detected Fragment; m/z	RT	Apple Juice A [a]		Apple Juice B		Apple Juice C		Apple Juice D		Apple Juice E		Apple Juice F	
			Original [b]	Evaporated [c]	Original	Evaporated	Original	Evaporated	Original	Evaporated	Original	Evaporated	Original	Evaporated
2-Methylpropyl acetate (**2**)	43	5.18	11.14	N.D. [d]	N.D.	N.D.	2.68	N.D.	4.30	N.D.	4.27	N.D.	0.21	N.D.
Ethyl butyrate (**3**)	71	5.77	3.74	N.D.	43.74	N.D.	3.00	N.D.	25.09	N.D.	10.16	N.D.	2.41	N.D.
Ethyl 2-methylbutanoate (**4**)	57	6.16	2.73	N.D.	0.10	N.D.	3.58	N.D.	4.56	N.D.	1.03	N.D.	0.24	N.D.
Butyl acetate (**6**)	43	6.59	210.26	0.06	6.50	0.06	96.17	0.06	99.99	0.09	61.94	0.07	8.07	0.06
Hexanal (**7**)	44	6.69	2.74	0.19	27.22	1.27	0.51	0.05	13.25	0.57	6.07	0.70	0.60	0.17
2-Methylbutyl acetate (**8**)	43	7.43	3.99	0.01	4.13	N.D.	5.00	N.D.	2.61	N.D.	0.72	N.D.	0.57	N.D.
1-Butanol (**12**)	56	8.24	105.63	1.42	24.83	0.88	15.07	N.D.	N.D.	N.D.	N.D.	N.D.	20.86	N.D.
2-(*E*)-Hexenal (**15**)	41	8.89	5.64	0.07	13.18	0.35	4.77	0.02	2.38	0.10	0.25	0.10	0.31	0.05
3-Methyl-1-butanol (**17**)	55	9.04	N.D.	N.D.	N.D.	N.D.	1.06	N.D.	1.37	N.D.	3.83	N.D.	N.D.	N.D.
Hexyl acetate (**19**)	43	9.49	37.84	0.01	0.92	0.01	17.07	0.02	19.80	0.02	11.42	0.02	0.89	0.02
Octanal (**22**)	43	9.87	0.07	0.03	0.12	0.03	0.34	0.06	N.D.	0.03	N.D.	0.08	0.09	0.07
1-Hexanol (**25**)	56	10.68	57.17	0.06	27.85	0.06	5.12	0.05	24.23	0.05	11.32	0.07	6.01	0.08
2-Methylbutanoic acid (**40**)	74	13.49	3.03	2.42	2.62	1.82	99.39	10.30	22.73	2.42	2.59	0.91	6.74	0.61

[a] A standard addition method was used for the quantification of volatile compounds in the apple juices.
Standard curves for each compound were composed of at least five points, corresponding to the relative intensities of each compound.
Values are concentrations of volatile compounds in apple juice and the concentration unit for all values is μmol/L.

[b] Original; original apple juice.

[c] Evaporated; apple juice from concentrate by evaporation.

[b] N.D., not detected.

samples for each compound and are shown in Table 3-2. The tendency found in Table 3-1 was not changed by the more accurate analysis. The selected compounds were detected at a high concentration in original juice C, and their concentrations were greatly reduced by evaporation.

3.3.3 Effects of volatile compounds in apple juice on the patulin production by *P. expansum*

The effects of the above-mentioned 13 volatile compounds on the patulin production by *P. expansum* were investigated using Evaporated C. The 13 compounds were added separately to Evaporated C at the same concentrations as those determined for Original C, as shown in Table 3-2 (original concentration), and also at the concentrations ten-times higher than those in Original C (ten-times concentration). Original C was used as the positive control and Evaporated C as the negative control.

Figure 3-2 demonstrates that the addition of butyl acetate (**6**), hexanal (**7**), 2-methylbutyl acetate (**8**), 1-butanol (**12**), (*E*)-2-hexenal (**15**), and octanal (**22**) did not affect the patulin production and that the concentrations of patulin in all cultures were not different from that of a negative control. By contrast, 2-methylpropyl acetate (**2**), ethyl butyrate (**3**), ethyl 2-methylbutanoate (**4**), hexyl acetate (**19**), 1-hexanol (**25**), and 2-methylbutanoic acid (**40**) increased the patulin concentration in the cultures. In cultures to which 2-methylpropyl acetate (**2**) or ethyl butyrate (**3**) was added at original concentrations, the patulin amounts in

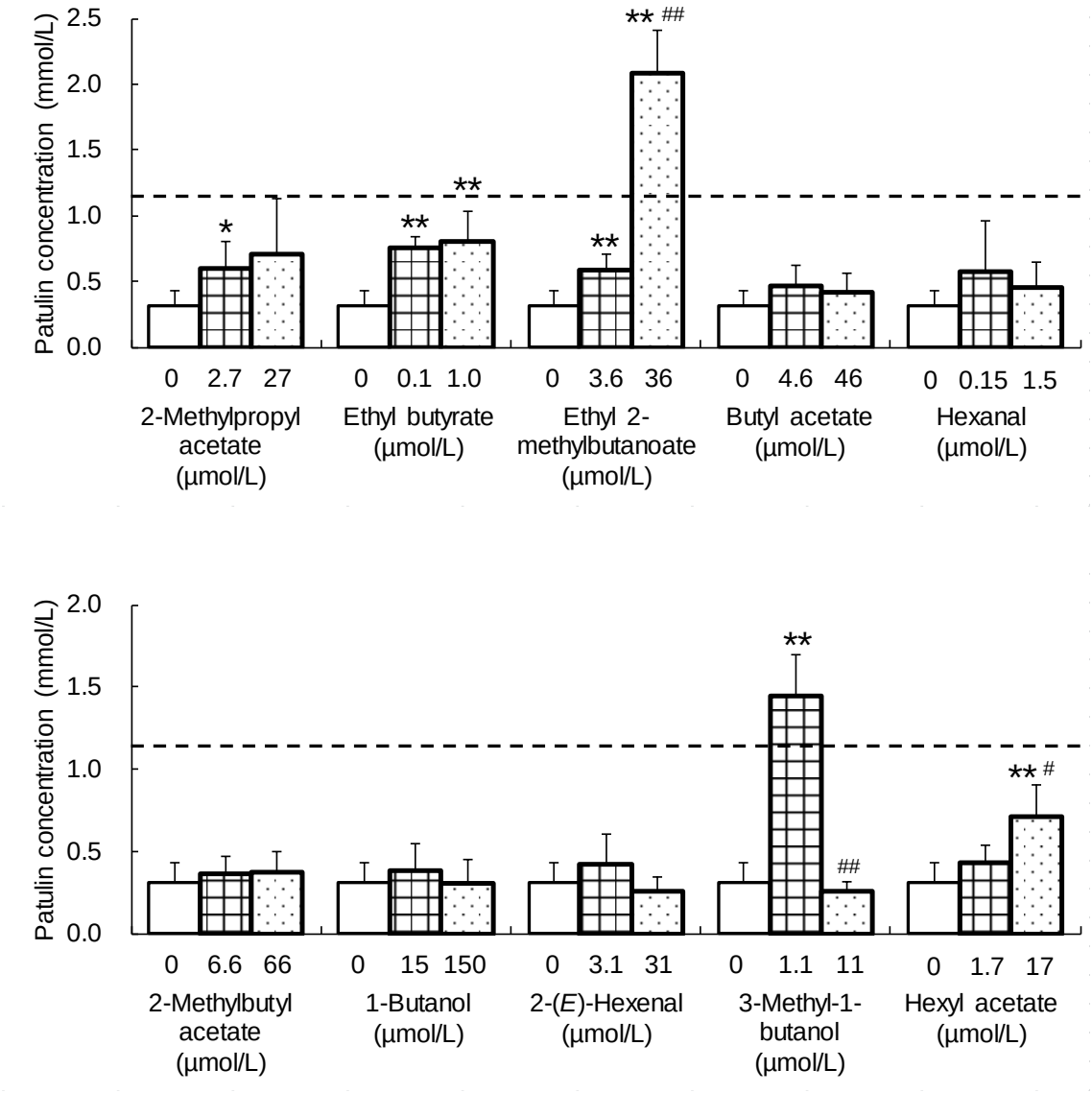

Fig. 3-2. Effects of the 13 volatile compounds on the patulin production by *P. expansum* in apple juice medium.

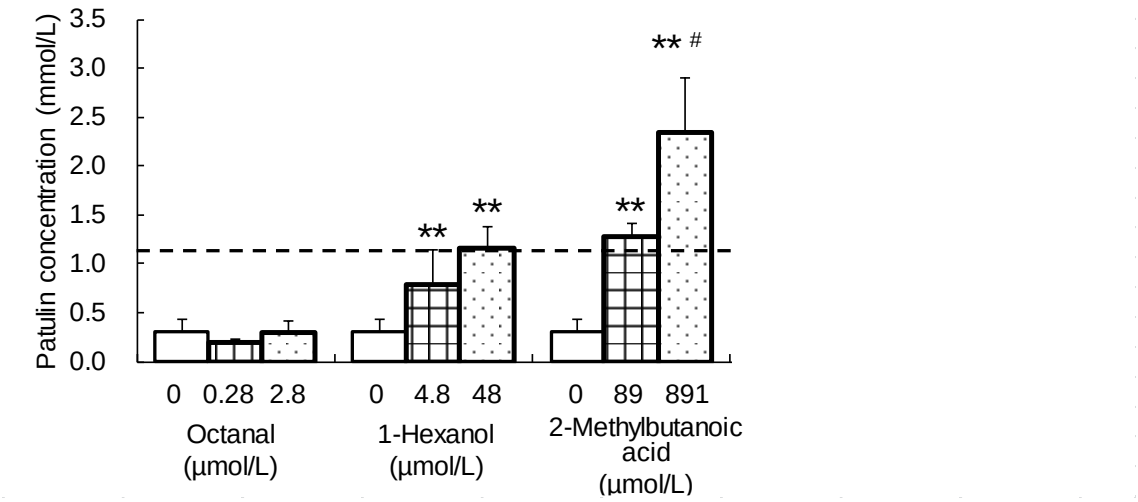

Fig. 3-2, Continued

The 13 compounds were added to Evaporated C. The final concentrations of compounds were adjusted to the same concentrations as Original C (center bars), and ten-times concentrations (right bars). Original C (dotted line) and Evaporated C (left bars) were used as positive and negative controls. The patulin concentrations in the graph are expressed as the means ± SD of five individual experiments. * $p < 0.05$, ** $p < 0.01$ compared with values for negative controls; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ compared with values for original concentrations.

the cultures were significantly increased compared to that in a negative control. However, when two compounds were added at ten-times concentrations, the patulin concentration in culture did not increase any further. The addition of ethyl 2-methylbutanoate (**4**) at the ten-times concentration, 3-methyl-1-butanol (**17**) at the original concentration, 1-hexanol (**25**) at the ten-times concentration, and 2-methylbutanoic acid (**40**) at both the original and ten-times concentrations stimulated the

patulin production up to or over the patulin level in Original C, whereas the addition of 3-methyl-1-butanol (**17**) at the ten-times concentration did not affect the patulin concentration.

3.3.4 Effect of 2-methylbutanoic acid on fungal growth and patulin production

Since 2-methylbutanoic acid (**40**) effectively stimulated the patulin production in Evaporated C, its effect on the patulin production by *P. expansum* was investigated in the other five apple juices. 2-Methylbutanoic acid was added to Evaporateds A to F at the same concentration as that in Original C, and at the concentration ten-times higher than that in Original C. Evaporateds A–F without adding 2-methylbutanoic acid were used as controls. A spore suspension of *P. expansum* was inoculated and incubated. After incubation, the dry weight of mycelia was measured as an indicator of fungal growth, and the patulin amount in the culture broth was determined by LC/MS (Fig. 3-3).

In the cultures of Evaporateds A, B, C, D and E, 2-methylbutanoic acid significantly increased the patulin amount and did so concentration-dependently, whereas in Evaporated F, a significant increase in patulin concentration was not observed. Conversely, in the A–F broths, the addition of 2-methylbutanoic acid significantly decreased the dry weights of mycelia of *P. expansum*. The relationship

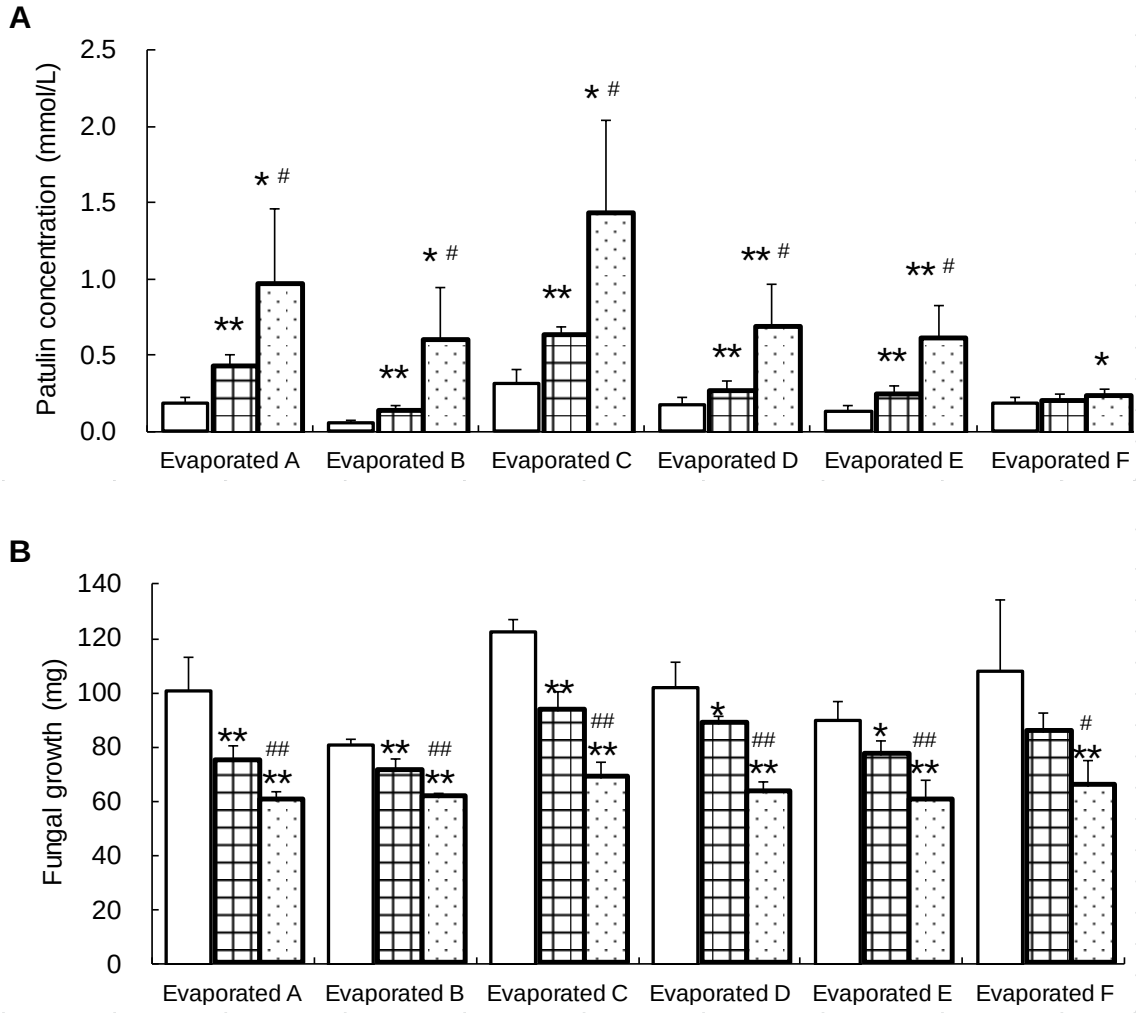

Fig. 3-3. Effects of 2-methylbutanoic acid on patulin production (A) and fungal growth (B) in six apple juice media.

2-Methylbutanoic acid was added to Evaporateds A–F. The final concentration were adjusted to the same concentration as Original C (89.1 mmol/L: center bars), and the ten-times concentration (891 mmol/L: right bars). Evaporateds A–F (0 mmol/L: left bars) were used as negative controls. The patulin concentration and fungal growth in the graph are expressed as the means ± SD of five individual experiments. * $p < 0.05$, ** $p < 0.01$ compared with values for negative controls; # $p < 0.05$, ## $p < 0.01$ compared with values for original concentrations.

between the decrease in the fungal growth and the enhancement of patulin production in apple juice broth by the addition of 2-methylbutanoic acid was the same as the relationship observed using from-concentrate apple juice broth. That is, the evaporation significantly reduced the patulin concentrations and significantly increased the dry weight of mycelia in apple juices A, B, and C.

3.4 Discussion

The present results demonstrate that patulin production by *P. expansum* depended on the broths made from the six different apple juices. Evaporation of these juices reduced the patulin production and simultaneouly increased fungal growth, suggesting that some volatile componds in these juices stimulate patulin production and inhibit fungal growth. In fact, seven volatile components (2-methylpropyl acetate, ethyl butyrate, ethyl 2-methylbutanoate, 3-methyl-1-butanol, hexyl acetate, 1-hexanol, and 2-methylbutanoic acid) of the apple juices were found to enhance the patulin production. In particular, since 2-methylbutanoic acid is relatively abundant and shows activity at a concentration in the original apple juice, it may play a key role in the patulin production by *P. expansum* in apple juice broths.

The addition of 2-methylbutanoic acid stimulated patulin production in the six different apple juices in a concentration-dependent

manner. A positive correlation (correlation coefficient 0.89) was observed between the 2-methylbutanoic acid concentration and the patulin production in the six Originals and six Evaporateds (Fig. 3-4).

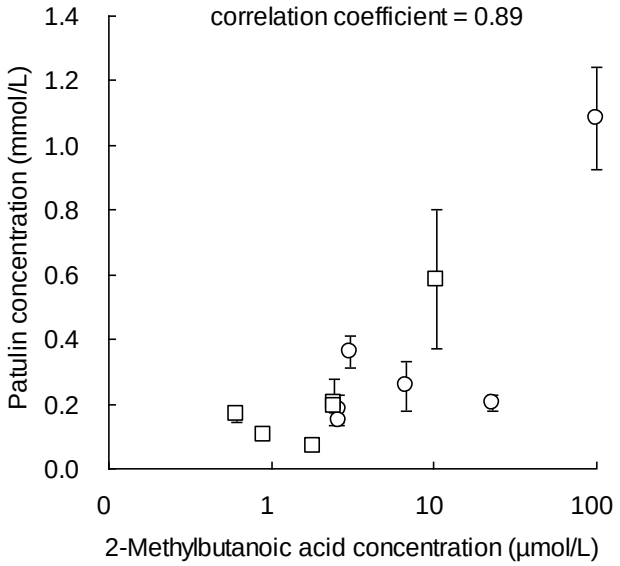

Fig. 3-4. The relationship between the 2-methylbutanoic acid concentration and the patulin concentration in the apple juice broths.

The correlation of the 2-methylbutanoic acid concentrations with the patulin concentrations in 12 culture media after the incubation of *P. expansum* in six Original broths (○) and six Evaporated broths (□) at 25°C for 7 d. The calculated correlation coefficient was 0.89. Symbols and error bars: means ± SD of five individual experiments.

This indicates that 2-methylbutanoic acid played an important role in the patulin production by *P. expansum* in the six apple juices used. However, the data for Original D largely departed from this correlation; although the concentration of 2-methylbutanoic acid was 22.7 μmol/L, the patulin amount was 0.20 mmol/L. Adding 2-methylbutanoic acid to Evaporated D resulted in an effective enhancement of patulin production, suggesting that Original D contains the volatile components that inhibited the patulin production by *P. expansum*.

The addition of ethyl 2-methylbutanoate also stimulated the patulin production in Evaporated C at a lower concentration than that of 2-methylbutanoic acid, indicating that ethyl 2-methylbutanoate has a greater effect on patulin production than 2-methylbutanoic acid. However, a correlation between the ethyl 2-methylbutanoate concentration and the patulin production was not found (correlation coefficient 0.47). These results suggest that a number of components of apple juices are involved in the regulation of patulin production by *P. expansum*, and their interactions are likely to be complicated.

The stimulation of patulin production by 2-methylbutanoic acid and its ethyl ester, both of which are enzymically biosynthesized from L-isoleucine in apple,[2,3] indicates that the 2-methylbutanoyl group might be an essential structural unit to elicit the enhancement of patulin production by *P. expansum*. Ethyl 2-methylbutanate was more effective for the enhancement of the patulin production than 2-methylbutanoic

acid, suggesting that the stimulating activity of these volatile compounds is affected by their hydrophobicity and permeability. It is of interest to analyze the structure-activity relationship among 2-methylbutanoic acid derivatives.

Manganese was reported to play an important role in patulin production by *P. expansum*.[4)] I thus suspected that there were significant differences in the contents of metals among the apple juices, and I determined the contents of metals in the six juices. Potassium, phosphorous, sodium, magnesium, calcium, and manganese were detected in all of the apple juices, but there was no correlation between the content of these metals and patulin production (data not shown), indicating that these metals are not the key regulatory factors for the patulin production by *P. expansum* in at least these apple juices.

The present findings demonstrated that at least five compounds other than 2-methylbutanoic acid and its ethyl ester stimulated patulin production. In apple juice, they may interact with each other in the stimulation of patulin production, sometimes synergistically and sometimes additively. The identification of such interactions would contribute to the development of methods to prevent the patulin contamination of apples and apple products.

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Chapter 4

4.1 General Disccusion

The effect of plant volatile compounds on patulin production by *Penicillium expansum* was determined by adding these compounds to apple juice broth. 2-Propenal, (*E*)-2-butenal, (*E*)-2-pentenal, and (*E*)-2-hexenal completely inhibited the growth of *P. expansum* and also inhibited patulin production by *P. expansum*. Their minimal inhibitory concentrations were 89, 713, 950, and 815 μmol/L, respectively. Vital staining indicated that these alkenals killed mycelia within 4 h, and the treatment of the spores with these aldehydes also resulted in a rapid loss of germination ability, within 0.5 to 2 d. These alkenals have a common structure: six or fewer carbons and an *E* double bond at the α-position, except for 2-propenal, which has no stereoisomers.

Gaseous (*E*)-2-hexenal was reported to be effective in the prevention of the contamination with *P. expansum* for pears.[1,2] and this is consistent with the present study's findings. The exposure of apples infected with *P. expansum* to these volatile alkenals as a vapor may thus be effective for sterilizing the fungus. However, because more effective compounds (i.e., 2-propenal and (*E*)-2-butenal) are known to have toxicity to humans, a process to remove vapor alkenals from apples is needed after sterilization.[3,4]

In the present experiments, several volatile compounds stimulated patulin production by *P. expansum* in a concentration-dependent manner.

For example, some aliphatic aldehydes, such as octanal, increased the patulin production without affecting fungal growth. The most effective concentration of octanal on patulin production was 2.3 mmol/L, whereas the maximum concentration of octanal observed in the six commercial apple juices used in this study was only 0.3 μmol/L. This indicates that octanal in apple juice does not stimulate the patulin production by *P. expansum*. In contrast, 2-methylbutanoic acid and its ethyl ester of apple juice stimulated the patulin production by *P. expansum* at their actual concentrations, i.e., 99.4 μmol/L and 3.6 μmol/L, respectively.

In this study, the mechanism underlying the stimulation of patulin production by octanal was found to be the increase in the transcript of the *msas* gene encoding 6-methylsalicylic acid synthase, which catalyzes the first step in the patulin biosynthetic pathway. 2-Methylbutanoic acid and its ethyl ester, whose stimulation mechanism has not yet been established, may also increase transcripts of the patulin biosynthetic genes in *P. expansum*. Further investigations are necessary to clarify the stimulation mechanisms of patulin production by 2-methylbutanoic acid and its ethyl ester.

Ethyl 2-methylbutanoate is present in many types of fruit, and descriptions of its aroma include “fruity” and “sweet” aroma notes; it apparently contributes greatly to the aroma of many fruits, including apple.[5–9)] By contrast, the odor of 2-methylbutanoic acid is described as “cheesy” or “rancid,” and is very different from apples’ aroma.[7,10)] This

indicates that 2-methylbutanoic acid is not necessarily an important aromatic component in apples and apple juice.

Several maize lines are known to have resistance to *Aspergillus flavus* infection and aflatoxin contamination, and the elucidation of the resistance mechanism has been carried out by molecular breeding.[11] The present results demonstrate that the selective breeding of an apple cultivar that does not contain compounds that stimulate the patulin production by *P. expansum* and that simultaneously have little effect on apple aroma (such as 2-methylbutanoic acid) is a promising approach for reducing the risk of patulin contamination without the loss of sensory values. Both a reduction of the risk of health hazards and a reduction of economic losses could thus be achieved by such an approach.

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LIST OF PUBLICATIONS

CHAPTER 2

1. Taguchi, T., Kozutsumi, D., Nakamura, R., Sato, Y., Ishihara, A., Nakajima, H. Effects of Aliphatic Aldehydes on the Growth and Patulin Production of *Penicillium expansum* in Apple Juice. *Biosci. Biotech. Biochem.*, **77**, 138-144 (2013)

CHAPTER 3

2. Taguchi, T., Ishihara, A., Nakajima, H. Effects of volatile compounds in apple juices on the fungal growth and patulin production of *Penicillium expansum*. *Mycotoxins* in press.

Abstract

For apples and their related products, contamination with a mycotoxin, patulin, is one of the most important concerns for both the food industry and consumers. The food industries are thus eager to develop a technique to prevent patulin contamination and infection with *Penicillium expansum*, the most common fungus associated with patulin contamination in apples. The patulin contamination of apples is not yet adequately controlled. Toward the development of new approaches to control patulin contamination in apples, I studied the effects of plant volatile compounds on patulin production by *P. expansum* in apple juice.

It is now widely known that some plant aroma compounds have antibacterial and antifungal activities. In particular, several aldehyde compounds have been found to have potent effects against microorganisms. I thus examined the effects of 16 aliphatic aldehydes with 3 to 10 carbons on the growth and patulin production of *P. expansum*. When *P. expansum* spores were inoculated into apple juice broth, some alkenals, i.e., 2-propenal, (*E*)-2-butenal, (*E*)-2-pentenal, and (*E*)-2-hexenal, inhibited fungal growth and patulin production. Their minimal inhibitory concentrations were 89, 713, 950, and 815 μmol/L, respectively. Vital staining indicated that these alkenals killed mycelia within 4 h. Treatment of the spores with these aldehydes also resulted in the rapid loss of germination ability, within 0.5 to2 d. By contrast, aliphatic aldehydes with 8 to 10 carbons significantly enhanced the

patulin production without affecting the fungal growth: 2.3 mmol/L of octanal and 790 μmol/L of (*E*)-2-octenal increased the patulin concentrations in the culture broth by as much as 8.6- and 7.8-fold compared to that of the control culture, respectively. The expression of the genes involved in patulin biosynthesis in *P. expansum* was investigated in mycelia cultured in apple juice broth containing 2.3 mmol/L of octanal for 3.5, 5, and 7 d. The transcription of the *msas* gene, which encodes 6-methylsalicylic acid synthase (which catalyzes the first step in the patulin biosynthetic pathway) was remarkably high in the 3.5-d and 5-d-old cultures compared to the control. However, octanal did not increase the transcription of the *msas* gene in the 7-d-old culture or that of two other genes, *IDH* and the *peab1*, in culture. Thus the enhanced patulin accumulation with supplementation with these aldehydes is attributable to the increased amount of the *msas* transcript.

Although the most effective concentration of octanal on patulin production was 2.3 mmol/L, the maximum concentration of octanal in the six commercial apple juices purchased from grocery stores in Japan was only 0.3 μmol/L. That is, aliphatic aldehyde compounds at their actual concentrations in apple juice do not stimulate the patulin production by *P. expansum*.

I then cultured *P. expansum* in apple juice broths made from six different commercial apple juices. Fungal growth was generally not affected by the differences in apple juices, whereas the patulin

production was profoundly affected; the maximum concentration of patulin in one type of apple juice was 7.3-fold the minimum concentration in another. The six apple juices were concentrated by evaporation and filled up to the original volumes by adding Milli-Q water. *P. expansum* was cultured in the broths prepared from concentrated juices, and the patulin concentration and fungal growth were determined. The patulin production tended to decrease and the fungal growth tended to increase, suggesting that the volatile compounds promote patulin production and inhibit fungal growth. The volatile compounds in the apple juice were analyzed by gas chromatography/mass spectrometry, and 57 compounds were detected. The 13 major compounds were selected and their stimulation of patulin production was evaluated. Seven of the 13 compounds (2-methylpropyl acetate, ethyl butyrate, ethyl 2-methylbutanoate, 3-methyl-1-butanol, hexyl acetate, 1-hexanol, and 2-methylbutanoic acid) increased the patulin production by *P. expansum* concentration-dependently; 2-methylbutanoic acid and its ethyl ester were highly effective. These results indicate that the composition of volatile compounds in apple juice broths has significant effects on the patulin production by and the growth of *P. expansum*.

The present findings also demonstrate that the selective breeding of an apple cultivar that does not contain compounds that stimulate the patulin production by *P. expansum* is a promising approach for reducing

the patulin contamination. A risk reduction in the health hazards and a reduction of economic losses could thus be achieved by this approach.

要旨

リンゴやリンゴ製品におけるカビ毒、パツリンの汚染は、食品企業、消費者ともに最大の関心事の 1 つである。そのため、食品企業ではパツリン汚染防止およびその主原因となるカビ、*Penicillium expansum* の感染防除技術の開発に力を注いできた。しかし、リンゴのパツリン汚染はいまだ十分に制御できていない。そこで、リンゴのパツリン汚染を制御する新しい試みとして、リンゴ果汁での *P. expansum* のパツリン産生に対する、植物が産生する揮発性化合物の影響を調査した。

植物の香気成分の一部は、抗細菌および抗カビ活性を有していることが広く知られている。特に、幾つかのアルデヒド化合物は微生物に対して高い効果を有していることが見つかっている。そこで、炭素数 3-10 個からなる 16 種類の脂肪族アルデヒド化合物が、*P. expansum* の増殖およびパツリン産生に及ぼす影響を調査した。*P. expansum* の胞子を 2-プロペナール、(*E*)-2-ブテナール、(*E*)-2-ペンテナール、および(*E*)-2-ヘキセナールを添加したリンゴ果汁培地に植菌したところ、カビの増殖が阻害され、同時にパツリン産生も観察されなかった。各アルケナール化

合物のリンゴ果汁培地中での最小阻害濃度は、それぞれ 89、713、950、そして 815 μmol/L であった。生体染色の結果、これらのアルケナール化合物は 4 時間以内にカビ菌糸体を死滅した。同様に、これらのアルケナール化合物で胞子を処理したところ、0.5-2 日以内という短期間の処理で胞子が発芽能を失うことが判明した。一方、炭素数 8-10 個からなる脂肪族アルデヒド化合物はカビの増殖に影響を与えることなく、パツリン産生量を有意に増加することが判明した。2.3 mmol/L のオクタナールおよび 790 μmol/L の(*E*)-2-オクテナールは、無添加の対照に対して、培養液中のパツリン濃度をそれぞれ 8.6 倍、7.8 倍に増加した。続いて、2.3 mmol/L のオクタナールを添加した培地で 3.5、5、そして 7 日培養したカビ菌糸体における、*P. expansum* のパツリン生合成に関わる遺伝子の発現状況を調査した。その結果、3.5 日および 5 日培養した菌糸体では、生合成系路の第 1 段階で作用する 6-メチルサリチル酸合成酵素をコードしている *msas* 遺伝子の発現量が、対照と比べて明らかに増加していた。しかしながら、7 日培養した菌糸体では、*msas* 発現量の増加は観察されなかった。同様に、他の 2 種類の遺伝子、*IDH* および *peab1* の発現量は、7 日間の培養期間を通じて増加しなかった。これらの結果は、オク

タナールなど一部のアルデヒド化合物の培地への添加によるパツリン産生量の増加は、*msas* 発現量の増加に起因していることを示している。

パツリン産生の促進に最も効果を示したオクタナール濃度は2.3 mmol/L であったが、6 種類の市販リンゴ果汁に含まれるオクタナール濃度は最大でも 0.3 μmol/L であった。つまり、脂肪族アルデヒド化合物は、実際のリンゴ果汁中の濃度では *P. expansum* のパツリン産生を促進しないことが判明した。

そこで続いて、6 種類の市販リンゴ果汁から調製したリンゴ果汁培地で *P. expansum* を培養したところ、リンゴ果汁の違いによるカビ生育への影響は小さかった。対して、パツリン産生へのリンゴ果汁の違いによる影響は大きく、最もパツリンが産生された果汁中のパツリン濃度は、最も低かった果汁中の 7.3 倍であった。さらに、各リンゴ果汁を減圧濃縮処理した後、ミリ Q 水を加えて還元処理した濃縮還元リンゴ果汁から 6 種類の培地を調製して *P. expansum* を培養し、カビの生育とパツリン産生に及ぼす影響を調べた。その結果、全てのリンゴ果汁において、濃縮還元処理によってパツリン産生量は減少し、カビの生育は促進される傾向が観察された。このことは、揮発性化合物がパツリン産生を促進し、カビの

生育を抑制することを示唆している。そこで、リンゴ果汁に含まれる揮発性化合物を GC-MS で分析したところ、57 化合物が検出された。そのうち、濃縮還元処理によって最も濃度が減少した 13 化合物を選択し、パツリン産生の促進効果を調べたところ、2-メチルプロピル酢酸、エチル酪酸、エチル 2-メチル酪酸、3-メチル-1-ブタノール、ヘキシル酢酸、1-ヘキサノールおよび 2-メチル酪酸の 7 化合物は、濃度依存的に *P. expansum* のパツリン産生を促進し、特に 2-メチル酪酸とエチル 2-メチル酪酸の 2 化合物はその効果が高かった。これらの結果は、*P. expansum* のパツリン産生は、リンゴ果汁中の揮発性化合物の組成の影響を大きく受けることを示している。

本研究の結果は、*P. expansum* のパツリン産生を促進する化合物を除いたリンゴの品種を選抜育種することで、パツリン汚染の減少を達成できることを示している。これが達成されれば、リンゴおよびリンゴ果汁のパツリン汚染による健康危害の減少や経済的損失の抑制が可能となる。