Production of Patulin by *Penicillium expansum*

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Twenty-seven isolates of *Penicillium expansum* Lk. ex Thom obtained from Europe, Australia, and North America from seven different fruit hosts all produced patulin in culture. Six isolates were essentially nonpathogenic in apple fruits. In culture, patulin generally accumulated to much higher levels than in apple fruits. At all temperatures permitting fungus growth, patulin was produced. However, only small amounts were observed near the maximal temperature for growth (30°C). At 0°C, patulin accumulated but slowly in culture. Modified atmospheres suppressed both fungus growth and patulin accumulation in apples. After varying incubation periods to obtain similar total growth, the patulin concentration was low in modified atmospheres and high in air.

The mycotoxin patulin is produced by several fungi, mostly species of *Aspergillus* and *Penicillium*. Included is *Penicillium expansum*, the blue-mold fungus of deciduous fruits. Its ability to grow at 0°C makes it a major cause of loss of apples and pears in storage. Other common fruit hosts include most *Prunus* sp., grapes, and persimmons.

Although patulin is acutely toxic to humans, its acute toxicity is believed unimportant because rotted flesh is easily avoided in fresh fruits and rotted fruits are eliminated before processing, thereby limiting ingestion. Long-term health problems from patulin-contaminated foods were suggested, however, when rats developed sarcomas after subcutaneous injections (5), although malignancies have evidently not resulted from oral ingestion (6, 11). Because patulin is heat stable (8), normal heat processing of fruits does not destroy it.

Patulin has been found in apple fruits (2, 7) and in apple juice (16, 17). Although Brian et al. (2) reported that some apple lesions did not contain patulin, Wilson and Nuovo (17), using a more sensitive detection procedure, found patulin in all of 60 cultures tested (all isolated from apples produced in a single orchard in Vermont).

Most studies of patulin production have utilized isolates from apples. Patulin production possibly varies considerably among isolates from different hosts and widely separated regions. This study was done, therefore, to compare patulin production in vitro and in vivo by isolates from different fruit hosts and widely separated regions. An additional objective was to determine the effects on patulin production of the temperatures and modified atmospheres used in fruit storage and transit.

**MATERIALS AND METHODS**

*Cultures.* Except as indicated otherwise, we isolated fungi from blue-mold lesions of deciduous fruit hosts and at locations shown in Table 1. Cultures were maintained on potato-dextrose agar slants stored at 7°C. To determine their capacity to produce patulin in culture, each isolate was grown in potato-dextrose broth prepared from fresh potatoes. After 600 ml of medium was dispersed in cotton-plugged, 2.4-liter reagent bottles, it was autoclaved for a time (60 min at 121°C) sufficient to produce a golden yellow color as suggested by Norstadt and McCalla (13). After inoculation, the bottles were placed on their sides and incubated at 22 to 24°C without agitation. Portions (15 ml) of broth were withdrawn aseptically from beneath the mycelial mats after 6, 9, 14, 16, 20, and 23 days and extracted. Patulin was estimated by inhibition of *Bacillus megaterium* as described below.

The pathogenicity of the isolates to Golden Delicious apple fruits was determined by stabbing opposite sides of each fruit to a depth of 2 to 4 mm with a needle covered with conidia. Fruits were incubated at 23°C in nonsealed polyethylene bags humidified by insertion of several wet paper towels. Relative pathogenicity was related to diameter of lesions after 6 days. Patulin levels in disease lesions were determined at 10 days of incubation after inoculation as above. Fungus-invaded tissue of lesions was removed and weighed, and patulin was extracted as indicated below.

*Extraction.* Patulin was extracted from 15-ml portions of culture broth by adding an equal volume of ethyl acetate, hand shaking for 2 min, centrifuging for about 2 min to hasten phase separation, and decanting the ethyl acetate. The extraction was twice repeated, and the ethyl acetate fractions were combined and dried over anhydrous MgSO₄ for 20 to 30
min. After filtering under slight vacuum, the extracts were evaporated to dryness under \( N_2 \), and the residue was dissolved in CHCl\(_3\) and adjusted to a convenient volume for analysis.

Patulin extraction from apple tissue utilized a modification of the method of Scott and Somers (15), who reported a 75 to 90% recovery of patulin from fruit juice. In brief, 54 g of lesion tissue plus 50 ml of water were blended, and the slurry was transferred to centrifuge tubes and extracted thrice as described above. After filtering, the combined ethyl acetate fractions were passed through a chromatographic column (2 x 40 cm) packed with 25 g of silica gel (60 to 100 mesh). The column was washed with about 100 ml of ethyl acetate. All the eluate was collected, evaporated to dryness under vacuum, dissolved in CHCl\(_3\), and adjusted to 1.0 ml.

**Patulin determination.** Thin-layer chromatography generally followed suggestions of Scott and Somers (15). Samples (10 \( \mu \)litters) of variously diluted extracts were spotted on thin-layer chromatography plates coated with 0.25 mm of 60 F-254 silica gel (EM Laboratories, Inc., Elmsford, N.Y.) and containing manganese-activated zinc silicate, which fluoresces in ultraviolet light. Patulin was found at about \( R_f \) 0.35 after development in benzene-methanol-acetic acid (18:1:1, vol/vol/vol).

Patulin was first estimated by comparing the quenching of fluorescence under short-wave ultraviolet light with that of authentic patulin. Subsequently, the phenylhydrazone derivative was prepared by spraying the plates with 5% \( \text{NH}_2\text{OH} \) followed by 4% aqueous phenylhydrazine and heating for 2 to 3 min at 110 C. Biological activity at the patulin \( R_f \) was sometimes verified by scraping that area from thin-layer chromatography plates after ultraviolet examination. After extraction with CHCl\(_3\), the patulin was transferred to a 6-mm disk prepared from Whatman 3MM paper and assayed by inhibition of growth of *B. megaterium*, a technique suggested by Clements (4) for assay of aflatoxins. Inhibition zones were compared with a standard curve previously established with authentic patulin. Diameters of zones of inhibition were near a straight line between 1 and 60 \( \mu \)g when concentrations were on the logarithmic scale of a semilog plot and diameter was on the arithmetic scale.

**Temperatures.** To determine the effect of temperature on mycelial growth and patulin accumulation, isolate 17 was grown in potato-dextrose broth. A sample (50 ml) of medium was autoclaved for 60 min at 121 C in cotton-plugged 125-ml Erlenmeyer flasks. After inoculation, the flasks were incubated at 25 C for 24 h before transfer to constant-temperature rooms (\( \pm 1 \) C). Forty flasks were incubated without agitation at each of the following temperatures: 0, 5, 10, 15, 20, 25, and 30 C. At intervals of 3 days to 2 weeks, depending on the temperature, 15-ml portions of broth were removed from each of two flasks for patulin analysis as described above. The mycelial mats were transferred to tared Buchner funnels and washed with the aid of vacuum. After drying for 24 h at 60 C, growth was recorded as milligrams (dry weight)/per milliliter.

**Modified atmospheres.** Air or an artificial atmosphere was passed through a 4-liter glass jar containing five apples inoculated with *P. expansum* isolate 17 or 24 as described previously. A flow rate of 25 to 300 cm\(^3\)/min prevented appreciable atmosphere changes due to fruit respiration. Artificial atmospheres were prepared by mixing flowing air with tank \( \text{CO}_2 \), and/or \( N_2 \). Gas flows were controlled with capillary flow meters as described by Claypool and Keefer (3).

**RESULTS AND DISCUSSION**

Concentrations of extractable patulin varied from less than 10 \( \mu \)g to nearly 1 mg/ml when cultures were grown in vitro (Table 1). Patulin concentrations were much lower in the water-soaked tissue of fungus lesions than in culture, varying from 2 to 125 \( \mu \)g/g (fresh weight). No isolate of *P. expansum* failed to produce patulin. Isolates that accumulated the highest quantities in vitro were not necessarily the highest when grown in vivo. For example, isolate 3 accumulated only 2 \( \mu \)g/g in vivo versus 330 \( \mu \)g/ml in culture. On the other hand, isolate 27 accumulated less than 10 \( \mu \)g/ml in culture but had a concentration of 50 \( \mu \)g/g in apple tissue. In addition, six isolates obtained from fruits other than apple were so weakly pathogenic in apples that no in vivo determinations were possible.

Observations that patulin is a phytotoxin, being implicated in apple replant problems (1) and poor growth of seedling wheat (12), suggest that it may play a role in the pathogenicity of *P. expansum* to deciduous fruits. In our tests, however, patulin concentrations in vivo did not relate closely to pathogenic vigor. The most vigorous pathogens were isolates 7 and 8, from Queensland and Michigan, respectively, but the lesions contained only 7 \( \mu \)g/g. On the other hand, isolates 6, 11, 12, and 16 contained 80 \( \mu \)g/g in lesions but were only about average in pathogenicity.

In studies on the influence of temperature, growth of *P. expansum* (isolate 17) reached a maximal dry weight of 0.17 g/flask (3.7 mg/ml) at all temperatures (Fig. 1 and 2). This maximum was reached in 2 to 3 weeks at 20 to 30 C but required 16 weeks at 0 C. Patulin accumulated to about 0.6 to 0.7 mg/ml in flasks incubated at 0 to 20 C. At 30 C, near the highest temperature permitting fungus growth, the maximal fungus growth was attained within 3 weeks, but patulin never exceeded 0.07 mg/ml. Hence, 30 C is presumably much less favorable for patulin accumulation than for fungus growth.

At 20 and 25 C, patulin levels decreased
Table 1. Patulin production and pathogenicity in apples of Penicillium expansum isolated from indicated host fruits and locations

| Isolate no. | Host     | Location              | Pathogenicity lesion diam (mm) | Patulin in vitro (μg/ml) | Patulin in vivo (μg/g) |
|-------------|----------|-----------------------|-------------------------------|--------------------------|------------------------|
| 1           | Apple    | Washington            | 33                            | 165                      | 35                     |
| 2           | Apple    | California            | 36                            | 140                      | 35                     |
| 3           | Apple    | California            | 32                            | 330                      | 2                      |
| 4           | Apple    | Washington            | 42                            | 850                      | 100                    |
| 5           | Apple    | California            | 25                            | 800                      | 10                     |
| 6           | Apple    | Ontario, Canada       | 28                            | 660                      | 80                     |
| 7           | Apple    | Queensland, Australia | 46                            | 660                      | 7                      |
| 8           | Apple    | Michigan              | 46                            | 470                      | 7                      |
| 9           | Apple    | Michigan              | 18                            | 15                       | 14                     |
| 10          | Crabapple| New South Wales, Australia | 4                     | 60                       | ND                     |
| 11          | Grape    | California            | 25                            | 560                      | 80                     |
| 12          | Grape    | California            | 26                            | 190                      | 80                     |
| 13          | Grape    | California            | 40                            | 60                       | 15                     |
| 14          | Grape    | California            | 1                             | 65                       | ND                     |
| 15          | Pear     | California            | 13                            | 30                       | ND                     |
| 16          | Pear     | California            | 20                            | 800                      | 80                     |
| 17          | Pear     | California            | 38                            | 950                      | 50                     |
| 18          | Pear     | California            | 29                            | 160                      | 3                      |
| 19          | Pear     | California            | 1                             | 30                       | ND                     |
| 20          | Apricot  | California            | 45                            | 610                      | 18                     |
| 21          | Apricot  | California            | 3                             | <10                      | ND                     |
| 22          | Apricot  | California            | 2                             | 40                       | ND                     |
| 23          | Persimmon| California            | 37                            | 660                      | 50                     |
| 24          | Laboratory Contaminant | California              | 30                            | 330                      | 125                    |
| 25          | Unknown  | England               | 42                            | 560                      | 45                     |
| 26          | Unknown  | England               | 36                            | 800                      | 14                     |
| 27          | Unknown  | France                | 15                            | <10                      | 50                     |

Data were obtained after the following incubation periods: lesion diameters, 6 days at 23 C; patulin in vitro, 21 days at 22 to 24 C; and patulin in apple fruits, 10 days at 23 C.

*Expressed as micrograms per milliliter of potato-dextrose broth.

*Expressed as micrograms per gram of fresh apple tissue.

*ND, Not done.

Fig. 1. Patulin production by Penicillium expansum (isolate 17) compared with fungus growth (mycelial dry weight) in potato-dextrose broth as influenced by temperature and incubation period.
strikingly after reaching peak levels (Fig. 1), suggesting a metabolic destruction. On the other hand, Pohland and Allen (14) showed that patulin gradually disappeared in aqueous solutions.

In limited tests, Gravenstein apples were inoculated with conidia of isolate 17. Patulin levels were compared in similar-sized lesions that had developed at 20 and at 0 C. After 5 days at 20 C, lesions were about 2 cm in diameter at the fruit surface and contained about 300 µg of patulin per g of fresh apple tissue. At 0 C, 36 days were required to obtain lesions of the same size that contained a maximum of about 100 µg/g. It is thus evident that storage at 0 C does not prevent patulin accumulation in apple fruits.

Modified atmospheres containing high concentrations of CO₂ (20 to 60%) reduced growth of Penicillium martensii and penicillic acid production in corn (10). High levels of CO₂ (20 to 100%) or low concentrations of O₂ (<5%) dramatically reduced growth of Aspergillus flavus and aflatoxin accumulation in peanuts (9). Because growth of P. expansum is also suppressed by atmosphere modifications, we tried to separate growth effects from patulin production. Incubation periods were varied to obtain disease lesions of approximately equal size (3.5 cm in diameter). Therefore, the increased incubation period in modified atmospheres to develop a lesion of that size is a measure of growth suppression (Table 2), which varied considerably among the isolates used. Either elevated CO₂ or reduced O₂, within the range tolerated by most living fruits (2 to 8% CO₂ and 2 to 3% O₂), suppressed patulin levels. Thus, patulin suppression was in addition to growth suppression. Since both growth and patulin accumulation are therefore suppressed, use of modified atmospheres in storage and transit might reduce the patulin hazard in deciduous fruits.

Table 2. Effect of atmosphere modification on patulin production by Penicillium expansum growing in Golden Delicious apples at 23 C

| Incubation period (days) | Atmosphere (%) | Patulin µg/g |
|-------------------------|----------------|--------------|
| P. expansum isolate no. 17 |                |              |
| 6          | Air            | 40           |
| 7          | 7.5 CO₂        | 24           |
| 8          | 2.0 O₂         | 2            |
| 8          | 2.0 O₂ + 7.5 CO₂ | 2         |
| P. expansum isolate no. 24 |                |              |
| 9          | Air            | 112          |
| 9          | 7.5 CO₂        | 12           |
| 20         | 2.0 O₂         | 4            |
| 20         | 2.0 O₂ + 7.5 CO₂ | 2         |

*Expressed as micrograms per gram of fresh apple tissue.

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