The Growth and Production of Patulin Mycotoxin by *Penecillium expansum* on Apple Fruits and its Control by the Use of Propionic Acid and Sodium Benzoate

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Abstract

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Penicillium expansum was the most frequently isolated fungal species in all tested samples of moulded apple fruits. The optimum temperature and RH for the growth of this fungus and for patulin production were 25^{o}C and 90%, respectively, and the high amount of patulin was expressed by isolate 3 at 18ºC (345.6 mg/ml^{-1}). The production of patulin correlated well with colony diameter and mycelium dry weight. Examined isolates varied in their inhibitory activity against *Escherichia coli* and *Bacillus subtilis* (isolate 3 had the best activity). The effect of propionic acid (0.05-0.2%) and sodium benzoate concentrations (0.05-0.4%) on the growth of *P. expansum* (isolate 3) and production of patulin from moulded apple surface were investigated. Propionic acid (0.15%) and sodium benzoate (0.3%) reduced the growth of the fungus and patulin concentration by more than 70%. Propionic acid at a concentration of 0.2 % and sodium benzoate at more than 0.3% almost prevented the growth of the fungus in the culture medium.

Keywords: *Penicillium expansum*, Patulin, Propionic acid, Sodium benzoate

Introduction

Penicillium is a large genus, with 150 recognized species, 50 or more of which are of common occurrence. All common species grow and sporulate well on synthetic or semi-synthetic media (22).

The blue mould agent *Penicillium expansum* is a common contaminant of apple in America and Europe (30), and is one of the most common pathogens for apple fruits in storage, transit and market (10, 34). It causes a soft rot of apples, the lesions being rather shallow at first but quickly become deeper, resulting in destruction of the whole fruit in 5-7 days at ambient temperature. Besides the economic loss associated with such storage pathogens, it has become clear, in recent years, that metabolic products of *P. expansum* may represent significant health hazards.

Apple tissue rotted by *P. expansum* contains the mycotoxin patulin (1, 3, 15, 24) which is also secreted by other species of *Penicillium*, as well as by *Aspergillus* and a number of other fungal genera (13, 29). This fungus produces mutagenic toxins and genotoxic secondary metabolites in apple (17) which can cause haemorrhaging in the brain and lungs, nausea, vomiting and gastrointestinal disturbance. In the United States, the Office of Environmental Health Hazard assessment has announced that patulin is a potential carcinogen (15). To reduce or eliminate such compounds, more information is required about factors affecting the formation of mycotoxins in plant tissue susceptible to fungal infection, e.g. suitability of fungal substrate, temperature requirement, moisture content and physical damage of apple fruits, as well as investigations of potential control treatments.

Different means have been used to control fungal contamination and their formation of toxins e.g. organic acids and their salts are known to be efficient against microorganisms, particularly moulds (4). These substances are generally used in conservation of food materials without leaving residues that may cause health hazards to the consumers (19). Their efficiency for inhibition of toxin production by fungi has been studied (27). Some of these chemicals were used successfully against new fungal strains, which had acquired resistance against fungicides (6). The prevention of mycotoxins in the environment is a big task; therefore, this research aimed to examine some environmental factors, e.g. temperature and relative humidity (RH), on the growth and production of patulin by *P. expansum* from apple and to gain an insight into the effect of propionic acid and sodium benzoate concentrations on the growth of this fungus and its capability for production of patulin.

Material and Methods

Isolation and Identification

The causal agent of the apple infection was isolated using the dilution technique (32). Isolation from the external surface was carried out by washing 1kg of apparently infected apple fruits of Golden Delicious variety, in 1 L of physiological water, containing 8.5g/l NaCl for each of the 5 samples, which were taken from the market randomly, shaken for 10 min in polyethylene bags and prepared to the

required dilution. Suspended spores in 0.1 ml samples from serial dilutions (10^{-1}, 10^{-2}, … 10^{-5}) were streaked on potato dextrose agar (PDA).

Tissue samples, 2-5 mm diameter, were cut from the edge of infected lesion of the apples, sterilized for 30 sec. with 10% sodium hypochlorite solution, washed in three changes of distilled water then placed on PDA in Petri-plates and incubated at 25°C. Fungal isolates were purified by subculturing on PDA.

Identification was based on the procedure of Pitt and Hocking (32). Growth was compared on (a)-Czapek yeast extract agar (CYA): 1.0 g dibasic potassium phosphate (K_2HPO_4), 10 ml Czapek concentrate, 5 g yeast extract, 30 g sucrose and 15 g Oxoid technical agar per 1000 ml of distilled water; (b)-25% glycerol nitrate agar (G25N): 0.75 g K_2HPO_4, 7.5 ml Czapek concentrate, 3.7 g yeast extract, 250 g glycerol and 15 g agar per 1000 ml of distilled water; and (c)-malt extract agar (MEA): 20 g malt extract, 1 g peptone, 20 g glucose and 15 g agar per 1000ml of distilled water at 5, 25 and 37°C. Colony size, color and microscopic characters were determined after 7 days.

Biological Assay Using *Escherichia coli* and *Bacillus subtilis*

Inhibitory activity against *E. coli* and *B. subtilis.* was compared in 14 isolates of *P. expansum* (25). Both species of bacteria were re-activated (grown) in nutrient broth, and incubated for 18 hr in 37ºC for *E. coli* and 30ºC for *B. subtilis.* 0.1 ml of each bacterial suspension (10^7 cells) was streaked on plates containing nutrient agar (NA) (Difco): 3 g peptone, 5 g NaCl, 15 g agar per 1000 ml of distilled water. 3 disks of 5mm diameter were cut from marginal regions of 7 days old fungal colonies grown on PDA**,** placed at an equal distance from each other, and from the center and the edge of the Petri-dish which contained the bacterial culture growing on NA. Plates were incubated first at 4°C for 24 hr, then transferred to 30°C for *B. subtilis* and 37°C for *E. coli* for 48h. The diameters of clearing zones, detected around the fungal disks, were recorded. The experiment was replicated 6 times. Isolate No.3 was chosen for the following experiments owing to its high pathogenicity, active degradation of the apple fruits and higher production of patulin.

The Effect of Temperature on Growth

Disks, 5 mm in diameter, were cut from 7-day-old cultures and inoculated in the center of 3 replicate agar plates containing PDA. The plates were incubated in the dark at different temperatures (5, 10, 15, 20, 25 and 30°C) and colony diameters were measured after 7 days.

The Effect of Relative Humidity on Growth

Different solutions of NaCl were prepared in sterilized distilled water in desiccators at 25°C, to give the following values of RH%: 80, 85, 90 and 95% (11). Disks of 5mm diameter were cut from a 7-day-old culture and inoculated in the center of 3 replicate plates of PDA, then incubated in desiccators at 25°C. The diameter of colonies was measured 7days later.

Extraction of Patulin

Inoculum disks (5 mm diameter) from fungal isolates, which revealed inhibitory activity against bacteria in the above test, by inducing clear zones of more than 20 mm diameter (33), were transferred to conical flasks (250 ml), containing 50 ml of yeast extract sucrose (YES): 20 g yeast extract, 150 g sucrose per 1000 ml of distilled water, and incubated at 18°C for 14 days. At the end of this period, fungal biomass was separated by filtration through Whatman No. 1 paper, then re-filtered through a membrane filter (0.45 µm porosity) to remove spores. The filtrate was washed with a similar volume of ethyl acetate (50 ml), shaken for 10min. and the organic phase then collected in a separate flask, dried with anhydrous Na_2SO_4, and washed with 25 ml of ethyl acetate. The solution was then reduced to almost dryness using a rotary evaporator (B-CH, RE121) at 48°C, re-dissolved in 2ml of chloroform and kept below -5°C until required for thin layer chromatography.

Thin Layer Chromatography

Qualitative separation of samples from extracts of each isolate, together with an authentic standard of patulin (Sigma, F69730), was carried out on silica gel TLC plates (20 x 20 cm, S & SD- Fertigfolien, F1500) using the solvent system toluene: ethyl acetate: 90% formic acid (5: 4: 1, V/V/V). The solvent was allowed to run to about 2cm below the top of the plate, which was then removed and left in the fume cupboard until the solvent was completely evaporated. The TLC plates were sprayed with 5% MBTH (3-methyl 2-benzothiaolionone hydrazine in distilled water), then dried at 130°C for 15 min, in order to detect patulin which appears as a yellow spot in visible light at RF= 0.44.

Quantitative estimation of patulin in YES culture, and from rotted apple fruit was carried out by a similar procedure. After the detection of patulin by UV (254nm), bands of equal width corresponding to the position of the standard were marked. Equal areas containing the separated patulin were transferred to test tubes. 5ml of 96% ethyl alcohol were added to each tube, mixed and shaken for 15min then centrifuged for 5min at 3000 rpm. The supernatant was poured into another tube, and the volume made up to 6 ml (35). Absorbance was read at 276 nm in a UV-Visible spectrophotometer (Shimudzu 1601). Absorbance values from the standard were used to prepare a calibration curve.

Estimation of Patulin in Apple Fruit

Golden Delicious apples were disinfected with 90% ethanol, and wounded midway (5 mm diameter x 3 mm deep) between the calyx and stem end axis with a sterilized cork borer. The wounds were inoculated with 20 µl of *P. expansum* conidial suspension (10^4 conidia per ml). Control treatments were inoculated with distilled water. The inoculated fruits were placed randomly on tray packs in plastic boxes containing moistened filter papers and incubated at 25°C for 15 days with 5 replicates. The lesion diameter was measured at 3 days intervals for 15 days (14). After the end of incubation period, 50 g of rotted tissue was removed, and added to 250 ml of ethyl acetate, homogenized for 10min with a glass homogenizer, and

procedures for the extraction and the estimation of patulin carried out as above.

The effect of Temperature on Patulin Production

Isolate 3 was grown on PDA for 7 days. Single disks (5 mm diameter) were then inoculated into three replicate conical flasks (250 ml) containing 50 ml of YES medium, and incubated in the dark for 14 days at 9, 18 and 25ºC. The extraction and estimation of patulin were carried out as described above.

The effect of propionic acid and sodium benzoate on *P. expansum*

To examine the efficiency of propionic acid and sodium benzoate for the control of *P. expansum* (isolate 3), a series of concentrations (0.5-0.20% for propionic acid and0.05-0.4% for sodium benzoate) were prepared in YES medium in 250ml conical flasks. Each flask was inoculated with a 5mm diameter disk from a 7-day-old fungal colony and incubated for 15 days at 25ºC. Dry weight of fungal mycelium was determined for each concentration and the amount of patulin in the medium was estimated as described above.

Results and Discussion

Identification of *Penicillium* isolates to species level, however, is preferably carried out under standardized conditions of media, incubation time, and temperature. In addition to microscopic morphology, gross physiological features, including colony diameters, colors of conidia and colony pigments, etc. are used to distinguish species (22).

In this investigation 18 isolates of *Penicillium* from separate apples were assessed and identified. Among these, 14 isolates were found to be *P. expansum*. All gave substantial clearing zones (more than 20 mm) bacterial growth suppression around the individual disks on both *E. coli* and *B. subtilis* (Table 1).

Temperature and relative humidity are considered among the most effective agents on the moulds growth and spores germination (23). It was found that isolate 3 of *P. expansum* in our study grows better in the temperature range from 15 to 25ºC. The optimum temperature and relative humidity for growth were 25ºC and 90%, respectively (Figures 1a and 1b).

The ability of the 14 isolates to produce patulin was examined under 18ºC. It was shown that all isolates secrete patulin with different quantity, which was characterized on TLC plates by yellow color and Rf= 0.44. The highest amount of patulin production was expressed by isolate 3 (Table 2). Isolates 3 produces greater amounts of patulin into YES medium (345.6 µg/ml) at 18ºC after 14 days and decreased to 139 µg/ml at 25ºC (Figure 3a). Doores (5) has reported that *P. expansum* secrets patulin more than 70 µg/ml at 12.8ºC in potato broth, and less amount at 7.22ºC. While, Land and Hult (16) have studied the ability of *P. expansum* to secrete patulin in the range of temperature from 4 to 25ºC in YES medium, and found that the amount is greater at 25 as compared to 4ºC. Podgorska (24) has studied the production of patulin by *P. expansum* in Czapek-Dox medium in a wide range of temperature varying from 5 to 30ºC, and found that the mould secreted the greatest amount of patulin at 25ºC from 2.2–2.4 mg per dm^3 culture liquid, and the smallest amount was secreted at 5ºC in 12 days. This suggests that the differences in patulin production depends on the culture conditions, medium composition, temperature, period of incubation and the used isolate.

Table 1. Effect of the metabolite isolates of *penicillium expansum* on growth of *Bacillus subtillis* (at 30C°) and *Echerichia coli* (at 37C°) as indicating by the diameter of clearing zones. Means of 6 replicates ± SD.

Isolates No.	Diameter of clearing zones (mm)	
	E. coli	***B. subtilis***
1	21.66 ± 1.10	28.33 ± 1.69
2	23.16 ± 0.68	33.50 ± 0.50
3	27.00 ± 1.00	33.66 ± 0.47
4	22.00 ± 1.52	29.16 ± 1.77
5	21.33 ± 1.79	28.00 ± 1.73
6	21.66 ± 0.74	27.33 ± 2.42
7	24.16 ± 0.68	32.83 ± 0.37
8	26.50 ± 1.50	30.00 ± 1.15
9	26.66 ± 0.94	32.50 ± 0.95
10	23.33 ± 0.94	31.16 ± 0.68
11	24.33 ± 1.69	33.50 ± 0.50
12	25.33 ± 1.88	30.83 ± 0.30
13	25.00 ± 1.00	30.33 ± 1.10
14	26.50 ± 1.50	29.33 ± 1.49

Table 2. Secretion of patulin by *P. expansum* isolates at 18ºC into YES medium. Means of 3 replicates ± SD.

Isolates No.	Patulin µg/ml	Isolates No.	Patulin µg/ml
1	336.0	8	327.0
2	237.0	9	100.0
3	345.6	10	256.0
4	324.0	11	130.0
5	207.0	12	177.0
6	313.0	13	125.0
7	140.0	14	225.0

The amounts of patulin diffused in apple fruit are well correlated with increasing lesion diameter. The production of patulin increased gradually after the sixth day of incubation, which was equal to 12.24% of lesion rate, and reaches 29.6 µg/g of rotted tissue, and rose up to 55.4 µg/g (50% of lesion rate) at 15 days (Figure 2). This amount of patulin in rotted tissue is near to that reported by Harwig *et al*. (12) which was 57 µg/g at 13-14 days, and agreed with the conclusion of Paster *et al.* (20) that there was higher production of patulin in apple fruit at 17ºC. On the other hand, patulin was found in apple products in amounts between 1-16400 µg/kg (8).

Propionic acid revealed high activity against *P. expansum*, a concentration of 0.2% (in distilled water) causing complete inhibition of the growth of this fungus.

Lower concentrations, 0.1% and 0.15% revealed a reduction less important, while 0.05% enhanced fungal biomass (Figure 3a). Suhr and Nielsen (31) investigated the inhibition of spoilage fungi in rye bread by using weak acid preservatives (concentration 0-0.3%) at a range of water activity and pH levels, and found that propionate was generally less effective than sorbate and benzoate at lower concentration, but 0.3% totally inhibited fungal growth. It was reported that 0.2% propionic acid minimized *Aspergillus flavus* throughout 12 days of experiment (2).

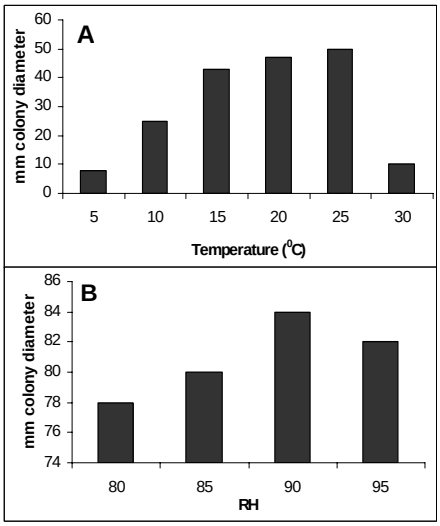

Figure 1. Effect of temperature (a) and relative humidity (b) on growth (colony diameter) of *Penicillium expansum* (isolate 3) on PDA, 7days. Means of 3 replicates ± SD.

Furthermore, Savard *et al*. (28) have observed on their study on two *Saccharomyces* sp a complete inhibition of growth with a mixture of lactic acid and priopionic acid of 0.7, 0.3 and 0.2%, respectively. Ghosh and Haggblom (9) observed that propionic acid reduced patulin production to 50% and to 96%, respectively, at concentrations of 0.05% and 0.15%. These concentrations also markedly reduced the production of aflatoxins. Marin *et al*. (18) found that suboptimal doses (0.03%) of all tested preservatives (potassium sorbate, calcium propionate and sodium benzoate) led to an enhancement of growth of *Aspergillus* and *Penicillium* isolates, but potassium sorbate was found to be the most effective in preventing fungal spoilage at the maximum concentration tested (0.3%).

Sodium benzoate (Figure 3b), at 0.05% in the medium was found to stimulate fungal growth, but concentrations of 0.25% and above reduced the growth of *P. expansum* to more than 50%. Patulin production was affected by 0.1%, but markedly reduced (50%) by 0.25%. The effect of sodium benzoate on fungal growth varied with isolates of *P. expansum* (24). It was demonstrated that 0.15% of sodium benzoate inhibited growth of *P. citrinum*, *Alternaria solani*, *Aspergillus niger* and the production of aflatoxin and growth of *A. flavus* and *A. parasiticus* decreased with increasing concentration of sodium benzoate (26). The mycelium mass growth correlated well with the ability of *P. expansum* to produce patulin (7). Our results agreed with those of Palou *et al.* (21) who found that potassium sorbate (0.2 M), and sodium benzoate (0.2 M) were the most effective organic acid salts among the wide range of chemicals tested and reduced gren mould by 70-80% at 7 days of storage at 20ºC. It can be concluded that both fungal growth and patulin production are affected by the level of propionic acid and sodium benzoate. However, propionic acid showed greater inhibitory activity against *P. expansum* than the latter. More investigation is still required.

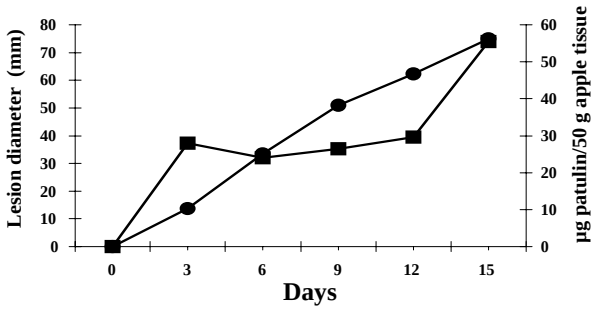

Figure 2. Lesion development (●) and the production of patulin (■) by *P. expansum* at 25 ºC for 15 days in apple fruits. Means of 5 replicates

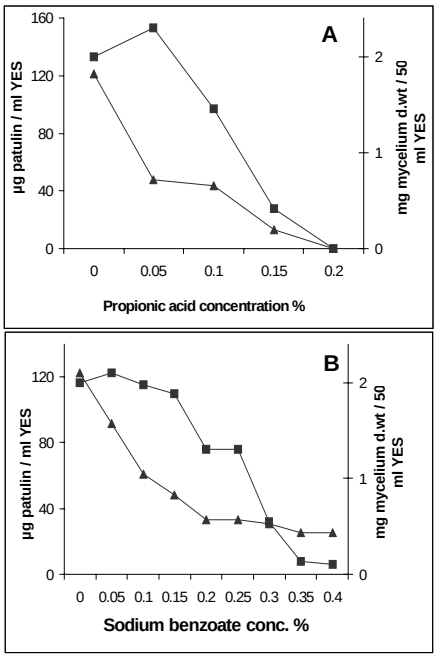

Figure 3. Effect of propionic acid (**a**) and sodium benzoate (**b**) on fungal biomass (■) and on patulin production (Δ) by *P. expansum* (isolate 3) at 25ºC in YES medium, 15days. Mean of 4 replicates ±SD.

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الملخص

لعروس، ل.، ن. هندل، ج.ك. عبود و م. غول. 2007. نمو الفطر *Penecillium expansum* على ثمار التفاح وإنتاج الفايتوكسين "باتولين" وطرق مكافحته باستخدام حمض البروبيونيك وبنزوات الصوديوم. مجلة وقاية النبات العربية. 25: 123−128.

تبين أن الفطر *Penicillium expansum* من أكثر الفطور ترددا والتي تم عزلها من ثمار التفاح المصابة بالعفن. لوحظ أن درجة الحرارة المثلى لنمو هذا الفطر وإنتاجه لمادة الباتولين هي 25°س والرطوبة النسبية 90%. الكمية القصوى لإنتاج مادة الباتولين وصلت من قبل العزلة 3 عتد درجة حرارة 18°س إلى 345.6 مغ/مل. كما تبين أن إنتاج مادة الباتولين مرتبطة بشكل كبير مع قطر مستعمرة الفطر والوزن الجاف للغزل الفطري. عند مقارنة قدرة العزلات المختلفة من الفطر *P. expansum* على منع نمو كل من *Escherichia coli* و *Bacillus subtilis* لوحظ أن العزلة 3 هي الأكثر تأثيراً. تم البحث عن تأثير تراكيز مختلفة من حمض البروبينيك (0.05−0.20%) وبنزوات الصوديوم (0.05−0.4%)على نمو الفطر *P.* expansum (العزلة 3) وإنتاج مادة الباتولين على سطح الثمار المتعفنة، وتبين أن المعاملة بحمض البروبيونيك بتركيز 0.15% أو بنزوات الصوديوم بتركيز 0.3% قد ساهمت في انخفاض نمو الفطر وإنتاج الباتولين بنسبة تزيد عن 70%، في حين أن حمض البروبيونيك بتركيز 0.2% أو بنزوات الصوديوم بتركيز 0.3% قد منعا نمو الفطر بشكل كامل على الوسط الغذائي.

كلمات مفتاحية: *Penicillium expansum*، الباتولين، حمض البروبيونيك، بنزوات الصوديوم.

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