ISSN 1110-0419

Annals Of Agric. Sc., Moshtohor,
Vol. 47(1): Fo. 13-22, (2009).

MOLECULAR CHARACTERIZATION OF *Penicillium expansum* AND ITS ROLE IN THE OCCURRENCE OF PATULIN IN APPLE JUICE

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ABSTRACT

This work was aimed to isolate and identifiy of patulin producer fungi and to follow the presence of patulin during apple juice processing. Among twenty two *Penicillium* isolates, eight isolates (two from healthy appeared apples and six from rot spotted apples) were considered as patulin producers using thin-layer chromatography. These isolates were classically identified as a *Penicillium expansum*. PCR utilizing primers based on the polygalacturonase gene of *P. expansum* was applied for detecting this mold. The PCR amplified a 404-bp DNA product from all tested *P. expansum* isolates, but not in other common food spoilage *Penicillium* species. RAPD technique using P1 or M13 primers was applied to determine the similarity of the *P. expansum* isolates. RAPD results revealed that the tested strains showed high percentage of similarity and no correlation was observed between cluster analysis and the sources of isolation.

Patulin could not be detected in healthy appeared apples and their extracted juice during different stages of juice process. In apple juice made from the healthy parts of apples decayed by *P. expansum* contained patulin which may present health hazard. The obtained results assured that patulin is known to be stable in apple juice even after pasteurization.

Key Words: Apple juice - *Penicillium expansum* – patulin – PCR – RAPD

INTRODUCTION

Postharvest spoilage of fruits results in shortened shelf-life and significant economic losses to the fruit industry. A variety of fungi are reported to be capable of producing patulin in defined media including *Aspergillus clavatus*, *A. giganteus*, *A. terreus*, *Byssochlamys fulva*, *B. nivea*, *Paecilomyces variotii*, *Penicillium carneum*, *P. clavigerum*, *P. concentricum*, *P. coprobium*, *P. dipodomyicola*, *P. expansum*, *P. glandicola*, *P. roqueforti*, *P. sclerotigenum and P. vulpinum* (Palmgren and Ciegler, 1983; Hasan, 2000). *Pencillium expansum* is a psychrotrophic mold and one of the most common fruit pathogens. It causes soft rot known as "a blue mold rot" on a variety of fruits, including apples, cherries, and peaches (Karabulut and Baykal, 2002; Vero *et al.*, 2002; Venturini *et al.*, 2002).

Besides its economic impact, *P. expansum* is also of potential public health significance since it is considered the major producer of patulin in fruit. The patulin is an unsaturated heterocyclic lactone, produced by certain fungal species of *Penicillium*, *Aspergillus* and *Byssochlamys* growing on fruit (Ritieni, 2003). Patulin has been mainly found in apple and apple products and occasionally in pears, grapes, apricots, strawberries, blueberries and peaches (Majerus and Kapp, 2002). Research papers in patulin toxicology showed that this mycotoxin may raise the risk

for the development of allergies (Wichmann *et al.*, 2002), can cause immunological, neurological, and gastrointestinal toxic effects in animal models (Bennett and Klich, 2003) and can induce DNA damage (Wu *et al.*, 2005). Codex alimentarius have recommended patulin concentration of <50 μg/kg for apple products intended for human consumption and the EU established a limit of 10 μg/kg for patulin in baby food and infant formulae (EC, 2003). On the other hand, The Joint Food and Agriculture Organization– World Health Organization Expert Committee on Food Additives has established a provisional maximum tolerable daily intake for patulin of 0.4 mg/kg of body weight per day (Trucksess and Tang, 2001).

Chen *et al.* (2004) evaluated the effectiveness of several chemical sanitizers against *P. expansum* NRRL 2304 to establish sanitizing wash treatments that would inhibit *P. expansum* growth and subsequent patulin production on Empire apples during cold storage. They stated that acetic acid solution (2% to 5%) was the most efficient chemical against *P. expansum*.

Recent and advanced papers were interested in the rapid methods for detection of patulin in juices and its fungal producers. Marek, *et al.* (2003) designed two primers for rapid and specific detection of *P. expansum* and its spores in apples. Also, Russell and Paterson (2006) suggested set of primers from the isoepoxydon dehydrogenase gene of the patulin biosynthetic pathway to indicate critical control points for patulin contamination of apples. Several methods for detection and determination of patulin in apple juice have been reported in reference literature. They include thin layer chromatography (AOAC, 1990; Paterson and Bridge, 1994; Paterson *et al.*, 2003), liquid chromatography (MacDonald *et al.*, 2000; Moake *et al.*, 2005; Iha *et al.* (2009), and gas chromatography/mass spectrometry (Cunha, *et al.* (2009).

Removal of decayed or damaged fruit or trimming moldy portions of apples prior to packaging or processing has been reported to reduce patulin levels in apple juice (Beretta *et al.*, 2000). Also, whole apples (table fruit) are not believed to contribute significantly to human exposure since contaminated fruit is often discarded or trimmed to remove moldy areas before it is eaten (Jackson and Dombrink-Kurtzman, 2006). In contrary, Taniwaki *et al.*, (1992) mentioned that total removal of patulin during the wash treatments is unlikely since patulin can diffuse up to 1 cm into the healthy tissue. For that reason, patulin is regularly found in applesauce and unfermented apple juice (Trucksess and Tang 2001). Therefore, FDA, (2004) stated that the greatest exposure to patulin comes from consumption of apple juice pressed from moldy fruit even after removing the moldy spots. This is a major concern since fruit juices, especially apple juice, are commonly consumed by infants and children. Therefore the present study was aimed to characterize patulin producer species in apple fruits and to follow occurrence of patulin during the production of apple juice.

MATERIALS AND METHODS

Apple fruits

Apple fruits were sampled from a commercial refrigerator in Alabour market, Alabour City, Egypt. The apples were divided into two groups (healthy-appeared and rot spotted apples), and stored at 4 °C during this study.

Fungal isolation and identification

Fungi were isolated from apple according to the method of Paterson *et al.*, (2003). For fugal isolation from healthy-appeared apples (25 samples), wash water from apple peel was obtained by pouring 10 ml sterile distilled water onto peel, and the wash water was plated directly onto Malt Agar (MA) with penicillin G and chloramphenicol (MApc, 75 mg l^{-1} MA) at 30 °C for 5 days. For fungal isolation from rotted apples (25 samples), small pieces (2x2 cm) selected from the margins of decay lesions from apple fruits were placed on MApc.

Pure cultures were obtained by transferring hyphal tips to MApc. Isolates were maintained on MApc at 4 °C, and were identified by macroscopic and microscopic observations. For identification of isolated fungi the book of Pitt and Hocking (1997) was used.

Extraction of apple juice

The infected spots were removed from the infected fruits manually by knife. Apple fruits (healthy-appeared and rot spotted apples) were washed by soaking in 2% acetic acid solution for 5 min with agitation at 100 r.p.m (Chen *et al.*, 2004) . The apples were milled, pressed and filtered through cheese cloth. The raw juice was treated with pectinases (Pectinex 100, 45 mg/l, and Pectinex Ultra, 10 mg/l) and amylases (AMG 300 L, 18 mg/l) from Novo Nordisk Biochem (Denmark), using dose of enzymes similar to the one used in the industry (Tajchakavit, *et al.*, 2001). The depectinisation was carried out at 50 °C ± 1 °C for 50 min. After that, the obtained juice was filtered through Whatman filter paper No.2 (Tajchakavit, *et al.*, 2001). The filtered apple juice was conditioned in glass bottle (200 ml) and pasteurized at 95 °C for 7 min by immersion in a hot water bath. The juices were then cooled under tap water. Samples were removed for analysis in duplicate after approximately 24 h of storage at 4 °C (Araya-Farias, *et al.*, 2007).

Detection of patulin by thin layer chromatography (TLC)

Patulin extraction

A modified version of the method used by MacDonald *et al.* (2000) was used for extraction of patulin from apple or apple juice. Ten grams of the sample were extracted twice with 20 ml of each using ethyl acetate by mixing vigorously for 1 min using a vortex mixer.

Fungal isolates were assessed for patulin production using a direct agar plug method from cultures grown on yeast extract sucrose (YES) medium at 30 °C until the diameter of fungal colony reached to about 5 cm. The mycelium was collected in a 50 ml falcon tube and weighted. Patulin was extracted from the mycelium by equal volume (w/v) of ethyl acetate.

Patulin detection

Ten, twenty and thirty microliter aliquots of sample extract and patulin standard solution (10µg/ml, Sigma, P-2639) were spotted 1cm apart on TLC plates (Whatman, TLC aluminum-back Kieselgel 60 plates). The spots were dried, and the plates developed in solvent system toluene: ethyl acetate: formic acid (5:4:1 v/v/v) (Labuda and Tancinová, 2006). For identification of patulin, the plates were sprayed with 0.5% aqueous methylbenzothiazolinone hydrazone hydrochloride monohydrate (MBTH) (No. 65875, Fluka, USA) and heated at 130 °C for 15 min. Patulin appeared as a yellow spot under visible light for reflection and transparency simultaneously and as a yellow–orange fluorescence spot under long wavelength (366 nm) UV light. The TLC plate was sprayed with water-90% formic acid (98:2 v/v) until the layer appeared wet and then observed under 366 nm UV light, which improved the visualization of the yellow–orange fluorescence spots against the background (Martins *et al.*, 2002)

Genomic DNA isolation and purification of fungal isolates

Fungal strains were cultured in 100-ml Erlenmeyer flasks containing 20 ml Mandles Andreoti medium (per liter: 10 g glucose; 2 g peptone; 2.8 g ammonium sulphate; 4 g KH_2PO_4; 10 g Na_2 HPO_4; 10 ml of a simplified Czapek's conc.; 7 g $MgSO_4$; 0.05 g $CuSO_4$-$5H_2O$; 0.1 g $FeSO_4$-$7H_2O$; 0.1 $ZnSO_4$-$7H_2O$; final pH adjusted to 5) for 5 days using a rotary shaker (30 °C, 150 rpm). The mycelium was collected by filtration and ground to fine powder in liquid nitrogen. Fifty milligrams of the powder was transferred to a 1.5 ml Eppendorf tube and mixed with 0.7 ml 2x cetyl trimethyl ammonium bromide (CTAB) buffer. The Eppendorf tubes were incubated at 65 °C for 30 min, and then 0.7 ml of chloroform was added and mixed briefly. After centrifugation at 15,000 rpm for 30 min, the supernatant was transferred into a new tube mixed with 0.6 ml isopropanol and chilled to 20 °C, followed by another centrifugation step for 5 min at maximum speed. The supernatant

was discarded and the remaining pellet was washed twice with 1 ml of 70% ethanol, followed by drying under vacuum and, thereafter, dissolved in 1 ml of TE (10 mM Tris, 1 mM EDTA, pH 7.5) buffer (Moeller *et al.*, 1992).

Detection of polygalacturonase gene of *P. expansum* by PCR

Forward (PEF, 5' ATCGGCTGCGG-ATTGAAAG 3') and reverse (PER, 5' AGTC-ACGGGTTTGGAGGGA 3') primers designned by Marek, *et al.* (2003) were used in the present study. These primers were selected from an internal, conserved sequence of the polygalacturonase gene of *P. expansum* published in the Genbank (Yao *et al.*, 1998; Accession number, AF 047713). These primers amplify a 404-bp DNA fragment on the basis of criteria described by Innis and Gelfand (1990).

A reaction mixture of 25 µl contained 200 µmol of each dNTP (Qiagen GmbH, Hilden, Germany), 250 nmol of each studied primer, 1 U Taq DNA polymerase (Qiagen), 2.5 µl of the buffer supplied with the polymerase and 0.5 µl of DNA template. The reaction was performed in a GenaAmp PCR system 9700 (Perkin Elmer Applied biosystems, USA) using the following temperature program (Marek, *et al.*, 2003). The reaction mixtures were heated at 92 °C for 5 min, then the PCR progressed for 30 cycles at a melting temperature of 92 °C for 1 min, annealing temperature of 55 °C for 45 s, and an extension temperature of 72 °C for 45 s. A final extension for 7 min at 72 °C was included at the end of the 30th cycle. After PCR, 10 µl of each PCR sample was analysed by electrophoresis in agarose gel (0.8 %) in 1xTAE at 80-100 V. The gel was stained for 10 min in 1xTAE containing 1 % ethidium-bromide and then visualized and photographed under UV illumination (Sambrook *et al.*, 1989). TriDye™ 2-Log DNA Ladder (0.1 - 10.0 kb) (New England Biolabs, Frankfurt, Germany) was used as a DNA molecular weight standard.

RAPD analysis

The PCR conditions and the separation of RAPD-PCR fragments were performed according to the techniques of Messner *et al.* (1994). The PCR' analyses were carried out with the aid of two different primers; P1 (5'- AAGAGCCCGT-3') and M13 (5'- GA-GGGTGGCGGTTCT -3'). PCR amplifications were performed in 50 µl volumes containing 1–1.5 units of Taq DNA polymerase (Qiagen) 200 µmol of each dNTP (Qiagen); 20 mM Tris-HCl (pH 8.4); 50 mM $MgCl_2$; 0.5 mM primer; and 15–20 ng of genomic DNA. Amplification was performed in a thermalcycler (GenaAmp PCR system 9700, Perkin Elmer Applied biosystems, USA) with the following temperature profiles: 98 °C for 5 min to denature genomic DNA; 40 cycles at 98 °C for 15 s; annealing at 40 °C for 90 s and extension at 72 °C for 100 s; followed by a final extension cycle at 72 °C. PCR products were resolved by electrophoresis as described previously.

RAPD data analysis

Computer analysis of RAPD patterns were performed as described by Halmschlager *et al.* (1995), in which the band pattern obtained from agarose gel electrophoresis was digitalized by hand to a two-discretecharacter-matrix (0 and 1 for absence and presence of RAPD bands, respectively) and the data of all primers were combined. The analysis data was based on the Nei and Li coefficient (Nei and Li 1979). A dendrogram was constructed by the unweighted paired group method of arithmetic average (UPGMA) based Jaccard's similarity coefficient by using Phoretex ID software (version 5.2).

RESULTS AND DISCUSSION

Fungal isolation

The presence of fungi on both healthy appeared and rot spotted apple is shown in Table 1. From 25 healthy appeared apple fourteen samples (56%) were infected with different fungi. Whereas all of rot spotted apple samples (100%) were infected. *Aspergillus niger* was the most commonly occurring

species on apple fruits (24 and 80 % on healthy appeared and rotted apple, respectively). This species is well known as a pathogen for many fruits (Agrios, 1988). *Cochliobolus spicifer, Gibberella fujikuroi, Mycosphaerella tassiana* were absent from tested healthy-appeared apple but present on the rot spotted samples.

From twenty two *Penicillium* isolates eight were identified as *Penicillium expansum*, two from the healthy appeared apples and six from the rot spotted apples (Table 1). *P. expansum* was previously isolated from apples decayed in refrigerated storage at 0 °C (Baert *et al.*, 2007).

Table (1): Fungal infection healthy appeared and rots spotted apple fruit samples

Fungus	Number of infected samples from			
	Healthy appeared apples (25 samples)		Rot spotted apples (25 samples)	
	No.	%	No.	%
Alernaria alternate	2	8	14	48
Aspergillus flavus	3	12	19	76
Aspergillus niger	6	24	20	80
Cochliobolus spicifer	0	0	1	4
Gibberella fujikuroi	0	0	1	4
Mucor racemosus	1	4	3	12
Mycosphaerella tassiana	0	0	1	4
Nectria hematoccoca	2	8	8	24
Penicillium expansum	2	8	6	24
Penicillium funiculosum	2	8	3	12
Penicillium oxalicum	3	12	4	16
Rhizopus stolonifer	2	8	4	16

Characterization of patulin producer isolates

Detection of polygalacturonase gene of P. expansum

PEF and PER primers designed by Marek *et al.*, (2003) were useful for detection of *P. expansum* isolates obtained in the present study (Fig. 1). The PCR product obtained by these two primers was 404-bp DNA fragment amplified from the chromosomal DNA of the eight *P. expansum* isolates (from both healthy appeared or rot spotted apples). On the other hand, no PCR products were obtained when the chromosomal DNA of other food spoilage *Penicillium* species were used as DNA template.

RAPD analysis

The primers P1 and M13 generated a considerable number of amplification products for comparison (Figs 2). These two primers typically generated between 12 and 19 DNA products for each *P. expansum* isolate. All amplification products were reproducible. Although the source of isolation of the eight *P. expansum* isolates was differed (healthy appeared and rot spotted apples), both primers (P1 and M13) showed high degree of similarity. Using *P. chrysogenum* as outgroup species a rooted tree was constructed from the combined RAPD date of both primers to show the relationships among the isolated strains (Fig. 3). The constructed dendrogram from the RAPD data showed two clusters. The first one was included P1, P2, P6, P7 and P8, whereas the second was included P3, P4 and P5. This means the *P. expansum* isolates (P1 and P2) obtained from healthy appeared apples were included in one cluster together with three *P. expansum* isolates (P6, P7 and P8) obtained from rot spotted apples. Therefore, it can be concluded that no correlation was observed between cluster analysis and the sources of isolation.

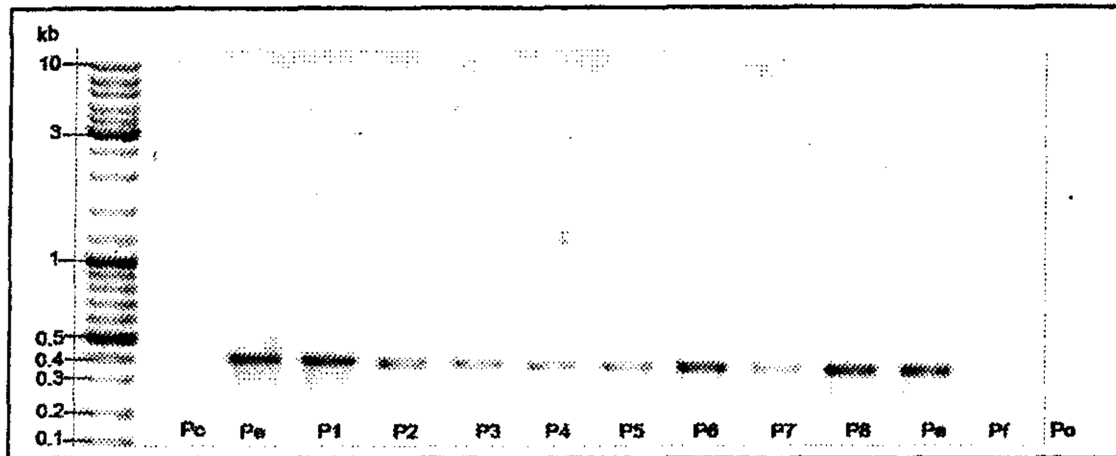

Figure (1): Agarose gel electrophoresis of PCR products from *Penicillium expansum* (lane P1 to P8) using PEF and PER primers. *Penicillium expansum* ATCC 28877 (lane Pe) and *Penicillium chrysogenum* NRRL 2273 (lane Pc) were used as positive and negative control, respectively. No PCR product was obtained by the other *pencillia* isolates *Penicillium funiculosum* (lane Pf) and *Penicillium oxalicum* (lane Po). TriDye™ 2-Log DNA Ladder (0.1 - 10.0 kb) was applied as standard DNA.

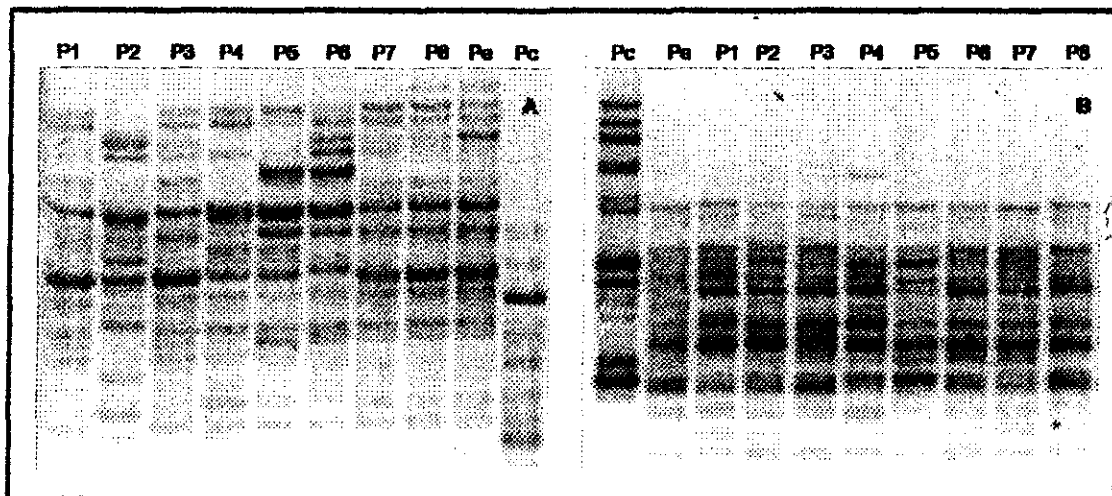

Figure (2): RAPD patterns obtained by using primer P1 (A) and M13 (B) of *Penicillium expansum* strains (P1 and P2 were isolated from healthy appeared apples; P3-P8 were isolated from rot spotted apples). *Penicillium expansum* ATCC 28877 (lane Pe) and *Penicillium chrysogenum* NRRL 2273 (lane Pc) were used as positive and negative control, respectively..

Patulin production by P. expansum isolates

Results of patulin detection by *P. expansum* isolates using TLC were summarized in Table 2. All *P. expansum* isolates grown on YES medium had ability to produce patulin. Moreover, patulin was detected on all rot spotted apples. Although, *P. expansum* isolated from health appeared apples produced patulin when they grown on YES, no patulin was detected in their source of isolation. This may be due to the surface contamination of healthy appeared apples with spores of *P. expansum*, which could be grown and produced patulin on the synthetic media, but not on the healthy appeared apples. In this respect, a report by Jackson, and Al-Taher (2008) indicates that the presence of a patulin-producing species does not necessarily imply patulin production in apples.

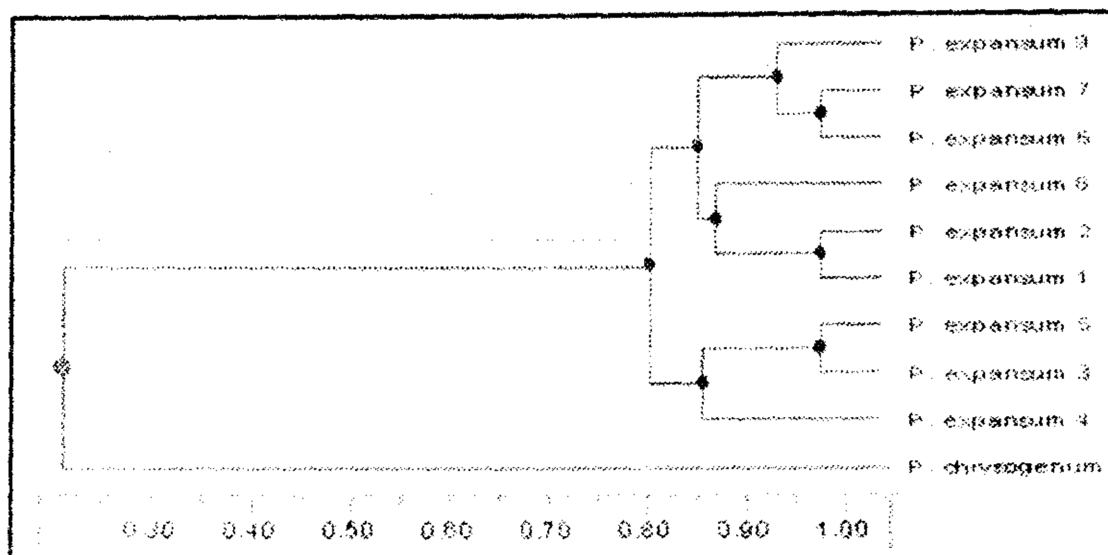

Figure (3): **Dendrogram showing the relationships among some *Penicillium expansum* strains depending upon the RAPD results using two different primers (P1 and M13)**

Table (2): **TLC detection of patulin by *P. expansum* isolates grown on YES medium and in healthy appeared and rot spotted apples**

Source of isolation	*P. expansum* isolates	Patulin production	
		By isolates grown on YES medium	In source of isolation (apple)
Healthy appeared apples	P1	+	-
	P2	+	-
Rot spotted apples	P3	+	+
	P4	+	+
	P5	+	+
	P6	+	+
	P7	+	+
	P8	+	+

Occurrence of patulin during apple juice processing

The occurrence of *P. expansum* and patulin on the healthy appeared and rot spotted apples and during juice processing stages was followed (Table 3). *P. èxpansum* and patulin were detected in rot spotted apples. Although *P. expansum* was detected in healthy appeared apples, patulin could not be detected. The processing stages including washing, milling and enzyme treatment had no effect on the presence of *P. expansum* and on occurrence of patulin. Wash process used in the present study, may be useful for inhibition of *P. expansum* growth and subsequent patulin production on apples during cold storage as recommended by Chen *et al.* (2004), but not to totally eliminate patulin formed in the fruits. Pasteurization process led to destroy *P. expansum*, however, patulin is remaining at the level of detection by TLC procedure des-crybed in the present study (Table 3).

The obtained results assured that, although removing visibly decayed fruit before processing is a proven method for reducing patulin levels in apple products, there is no guarantee that culling alone can totally eliminate patulin. In this respect, there is number of studies devoted to the removal of patulin from apple beverages and similar products during industrial scale production, therefore, beginning the extraction process with good and healthy fruits is really

significant to reduce chances of patulin occurrence in apple beverages (Beretta, *et al.*, 2000). In fact, commercial apple juices containing pulp analyzed by these authors showed a patulin concentration of 0.68–1150 mg/kg. Moreover, Jackson and Dombrink-Kurtzman (2006) mentioned that apples with "invisible" sources of fungal rot (core rots) can contaminate apple juice or puree with patulin.

Table (3): Effect of processing stages of juice production from healthy appeared and rot spotted apples on the occurrence of *P. expansum* and patulin.

Processing stage	Fruit status			
	Healthy appeared		Rot spotted	
	P. expansum	Patulin•	*P. expansum*	Patulin•
Raw apple fruits	+	-	+	+
After washing	+	-	+	+
After milling	+	-	+	+
After enzyme treatment	+	-	+	+
After pasteurization	-	-	-	+

* detected by isolation on MApc • detected by TLC

CONCLUSION

The obtained results demonstrated that (i) the removal of the rotten part of the fruit does not ensure the complete toxin elimination, because patulin could be present in the healthy part of rot spotted fruits. (ii) washing with water, milling and enzyme treatment were not sufficient to inhibit the fungal growth or remove patulin. (iii) even after pasteurization patulin is occurred in the apple juice processed from the healthy parts of rot spotted fruits this ensure that patulin is a heat stable mycotoxin.

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التوصيف الجزيئي لفطر بنيسيليوم إكسبانسيم ودوره فى وجود الباتيولين في عصير التفاح

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يهدف هذا البحث إلي عزل وتعريف الفطريات المنتجة للباتيولين الملوثة لثمار التفاح وتتبع وجود هذا السم الفطري خلال عمليه إنتاج عصير التفاح. من بين اثنتان وعشرون عزله من فطر البينيسيليم وجد أن ثمان عزلات منها لها القدرة علي إنتاج الباتيولين وقد تم التقدير باستخدام تقنية التحليل الكروماتوجرافي (TLC). هذه العزلات منها اثنتان تم عزلهما من السطح الخارجي لمجموعة ثمار التفاح السليمة وستة عزلات تم عزلها من أماكن الإصابة من مجموعة الثمار المصابة. وقد ثبت باستخدام تقنية تفاعل البلمرة المتسلسل (PCR) أن هذه العزلات تتبع فطر *Penicillium expansum* وذلك بالكشف عن جين بولي جلاكتيورينيز polygalacturonas حيث تكون ناتج التفاعل المحدد بـ ٤٠٤ قاعدة نيتروجينية والتي لم تتكون عند استخدام العزلات الأخرى من البينيسيليوم . وقد أوضحت تقنية التضاعف العشوائي للمادة النووية (RAPD) وجود درجة عالية من التشابه بين الثمان عزلات من *P. expansum* وقسمت الي مجموعتين. وأثبت تحليل هذه النتائج أنه ليس هناك علاقة بين اختلاف المجموعة ومصدر العزل.

وقد اتضح من النتائج أن ثمار التفاح السليمة خالية من الباتيولين بالرغم من وجود الفطر، كما انه لم يثبت وجود الباتيولين خلال المراحل المختلفة لإنتاج عصير التفاح من الثمار السليمة. وقد ثبت وجود الباتيولين في الأجزاء السليمة من الثمار المعطوبة (بعد إزالة الأجزاء المعطوبة) كذلك ثبت وجود الباتيولين خلال المراحل المختلفة لإنتاج العصير من الأجزاء السليمة للثمار المعطوبة حتى بعد إجراء عملية البسترة.

وقد خلصت الدراسة إلي ضرورة استبعاد الثمار المصابة وعدم استخدام الأجزاء السليمة منها في إنتاج عصير التفاح وذلك لتلوثها بالباتيولين الثابت حراريا والذي ثبت وجودة في العصير النهائي بعد البسترة