Quality Assurance and Safety of Crops & Foods, 2017; 09 (1): 23-29

Wageningen Academic
Publishers

Patulin risk associated with blue mould of pome fruit marketed in southern Italy

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Received: 20 October 2015 / Accepted: 19 March 2016

RESEARCH ARTICLE

Abstract

Blue mould is one of the most important postharvest diseases of pome fruit in all producing countries. It is mainly associated to *Penicillium expansum* that produces the mycotoxin patulin, although other species might be involved. The aim of the present study was to characterise *Penicillium* isolates associated with blue mould decay of pome fruit marketed in Apulia region (southern Italy), and verify their ability to produce patulin *in vitro.* Twenty-nine isolates of *Penicillium* spp. were recovered from pome fruit showing visible blue mould symptoms, and analysed for patulin production. After fungal isolation, the fruits were singularly analysed for patulin content. In general, the isolates proved to produce patulin and most of the pome fruit contained significant amounts of patulin, but there was no quantitative correspondence between *in vitro* and *in vivo* toxin accumulation. Isolate identification at species level was based on DNA analysis by *P. expansum* species-specific primers and sequencing of *β*-tubulin gene. Furthermore, fungal isolates were tested for the occurrence of the *patN* gene coding the enzyme isoepoxydon dehydrogenase (IDH), involved in patulin metabolic pathway and considered a useful indicator of critical control points for patulin contamination. All 26 isolates identified as *P. expansum* were positive for *patN* and produced patulin. Moreover, three pear isolates belonging to other *Penicillium* species were found. They were positive for *patN*, but only two actually produced patulin. It can be concluded that toxigenic *P. expansum* isolates are associated with blue mould of pome fruit marketed in Apulia, thus a rapid detection is important to avoid patulin contamination beyond regulatory limits. Nevertheless, the presence of *patN* gene alone cannot be considered a predictive assay for patulin production. An evaluation of its expression level should be carried out.

Keywords: *Penicillium*, mycotoxin, isoepoxydon dehydrogenase, apple, pear

1. Introduction

Penicillium expansum Link, a psychrotrophic mould and one of the most common fruit pathogens, is commonly identified as causal agent of blue mould of pome fruit, which are largely infected in warehouses and in the flume water of packinghouses (Sanzani *et al.*, 2009a). *P. expansum* infections are responsible for significant economic losses to the fruit industry, since they shorten fruit shelf-life and pose a risk for public health because of patulin accumulation. The natural occurrence of this mycotoxin has been associated with several fungal genera including *Penicillium*, *Aspergillus*, *Paecilomyces*, and *Byssochlamys* (Weidenbörner, 2001); however, it is mainly related to *P. expansum*. Initially isolated as a broad-spectrum antifungal antibiotic, during the 1960s patulin was reclassified as a mycotoxin, because of its mutagenic, immunotoxic, and neurotoxic properties (Iwahashi *et al.*, 2006), although no adequate evidence of carcinogenicity in experimental animals exists (Castoria *et al.*, 2012). As continued evidence for the negative health effects of patulin consumption, the European Commission (EC, 2006) established maximum permitted levels for this mycotoxin in fruity foodstuffs: 10 μg/kg for baby food, 25 μg/kg for purees, and 50 μg/kg for juices. Indeed, apples and derived products rank at the 17th place in the list of the highest produced commodities worldwide (FAOSTAT, 2012), and remain the major sources of patulin ingestion in humans (Moake *et al.*, 2005), although *P. expansum* ability to infect and extensively contaminate other fruits (grape, sweet cherries, etc.) should be taken into account (Sanzani *et al.*, 2013).

Several studies on patulin occurrence in fresh and derived products commercialised in Italy were conducted. In 2000, Beretta *et al.* analysed 26 apples, 21 of which proved to contain patulin in the range of 2-113 µg/kg. In another study, 11 out of 40 apple-derived products were contaminated in the range 16.7-74.2 µg/kg (Ritieni, 2003). In a larger survey conducted on 100 conventional and 69 organic fruity foodstuffs samples marketed in Italy, patulin was detected in 26% of conventional and 45% of organic products with a significantly higher (P<0.01) mean level in the organic products (i.e. 4.78 vs 1.15 µg/kg) (Piemontese *et al.*, 2005). In 2007, Spadaro *et al.* published a survey on the occurrence of patulin on commercial purees (53 samples) and mixed apple juices (82 samples) marketed in Italy. Mean patulin levels were significantly lower in mixed apple juices (4.54 µg/kg) than in pure apple juices (9.32 µg/kg). A recent small survey conducted on 80 samples of baby foods confirmed the significantly higher levels of patulin in organic products with respect to those from conventional production (Sarubbi *et al.*, 2016).

Therefore, although the use of undamaged fruits for juice production has been recommended, patulin has been constantly detected in several fruity foodstuffs. A possible explanation might come from the ability of *P. expansum* to live endophytically and produce patulin without visibly rot symptoms (Paterson *et al.*, 2000), or to penetrate through the natural openings causing internal rot in externally healthy fruits (CAC, 2003). Furthermore, as mentioned above, other fungi in the pome fruit production system could contaminate juice (Pianzzola *et al.*, 2004). *Penicillium* species identification by morphological features is notoriously difficult, and can result in misidentifications. Nucleic acid-based methods such as PCR amplification using species-specific primers or sequencing of barcode genes, provide powerful and rapid tools for the detection (Schena *et al.*, 2013). Moreover, they are useful in investigating the potential of fungi to produce mycotoxins by detecting genes involved in the biosynthesis, such as the *patN* gene that codes for the enzyme isoepoxydon dehydrogenase (IDH) involved in the conversion of isoepoxydon to phyllostine in patulin biosynthetic pathway (Sanzani *et al.*, 2009b). Although the cultivation of pome fruit is mainly associated to Northern Italy, recently it has become increasingly important in Apulia (Southern Italy), which has a cultivation area of 718 ha with an annual production of over 100,800 tons (ISTAT, 2011). In addition, an increasing number of companies in the region are dedicated to making products derived therefrom. Consequently, patulin could significantly affect the safety of pome fruit-derived products and economy of the region.

In the present study, *Penicillium* species associated with blue mould symptoms of apples and pears marketed in Apulia were characterised. Moreover, patulin production potential of the collected isolates was evaluated.

2. Materials and methods

Sampling

Apples (*Malus domestica* Borkh) and pears (*Pyrus communis* L.) were purchased from fruit and vegetable markets located in Apulia (Southern Italy). In particular, 11 pears of two common cultivars (Abate Fetel and Conference), 9 apples of two red cultivars (Starking and Annurca) and 9 apples of a yellow cultivar (Golden Delicious) were sampled.

Isolation of fungi

Pears and apples with visual evidence of *Penicillium* spp. contamination were placed in plastic bags and incubated in the dark at 24±1 °C for 7 (yellow apples), 14 (red apples) or 21 (pears) days. Conidia from each lesion were collected and suspended in 0.05% (v/v) Tween 80. Suspensions were spread on dishes of Dichloran Rose Bengal Yeast Extract Sucrose (DRYES) agar (Samson and Pitt, 2000), and incubated at 24±1 °C for 14 days. For each dish, the most prominent among the emerging colonies morphologically identified as *Penicillium* spp. was transferred to a Potato Dextrose Agar (PDA; Samson and Pitt, 2000) plate. All cultures were incubated for 7 days at 24±1 °C and purified as required. The monoconidial isolates were recorded as ITEM from 7005 to 7033 and deposited in the 'ITEM Agri-food Microbial Culture Collection' of the Institute of Sciences of Food Production (CNR, Bari, Italy; http://www.ispa.cnr.it/Collection).

Patulin determination

In vivo *determination*

After conidia collection, the fruits were singularly homogenised and a 10 g portion was analysed according to Mac Donald *et al.* (2000). Briefly, the puree was digested overnight at room temperature with 500 µl of a pectinase enzyme (1,350-1,650 U/g; Orsell S.R.L., Modena, Italy) in presence of 10 ml water and then centrifuged (Beckmann centrifuge, Allegra X 22; Fullerton, CA, USA) at 3,900×g for 10 min. The supernatant was transferred in a separatory funnel and extracted three times with ethyl acetate by liquid-liquid partitioning (LLP). The upper organic layers were collected and cleaned up by LLP with 1.5% sodium carbonate solution. The upper organic layer was collected and water deprived by passing through anhydrous sodium sulphate then evaporated in a rotavapor apparatus (Büchi Labortechnik AG, Flawil, Switzerland) set at 40 °C and 240 mbar, and re-dissolved in 4 ml of acidified distilled water (pH adjusted to 4 with pure acetic acid). Purified extracts were analysed by high performance liquid chromatography-UV-diode array detector (HPLC-UV-DAD) as reported below. Results were expressed as µg of patulin per kg of fruit puree (µg/kg).

In vitro *determination*

Each of the 29 isolates of *Penicillium* spp. was subcultured on five PDA plates (Ø 90 mm) for a total of 145 plates, and incubated for 21 days at 24±1 °C. Patulin was extracted from the mycelium of each plate with 3 ml of acidified distilled water by scraping with a spatula. For each isolate the water extracts collected from the 5 plates were pooled, filtered through two gauze layers, centrifuged (Beckmann centrifuge) at 3,900×g for 5 min at room temperature and finally filtered through a 0.45 µm syringe filter (Albet, Murcia, Spain). A 10 ml aliquot was extracted and purified as described above for purees, brought to dryness, resuspended in 4 ml acidified distilled water and analysed by HPLC-UV-DAD. Results were expressed as µg of patulin per cm^2 of plate surface ($\mu g/cm^2$).

HPLC analysis

Patulin analysis was performed by injecting 50 µl of the extract into a liquid chromatograph (ThermoQuest Inc., Parkway San José, CA, USA) equipped with: a quaternary gradient pump capable of delivering 1 ml/min constant flow rate (Spectraseries gradient pump P4000; Thermo Scientific, Waltham, MA, USA), a vacuum membrane degasser (SCM 1000; Thermo Scientific), an autosampler injection system with a 50 µl loop (AS 3000; Thermo Scientific), a column oven set at 30 °C, a UV-DAD detector (UV 6000 LP; Thermo Scientific) set at 276 nm, and a chromatography data system for Windows 2000 (ChromQuest version 2.53; Thermo Scientific). A Phenomenex C18 Synergi Hydro column (250×4.6 mm, 4 µm particle size) (Phenomenex, Torrance, CA, USA) preceded by a guard filter (3 mm, 0.5 µm pore size) was used. The mobile phase was a mixture of water, acetonitrile and perchloric acid (98:2:0.1, v/v/v). Patulin was identified by comparing the retention time and UV spectrum of the peak recorded in the sample chromatogram with those of an authentic standard. Quantitation of the toxin levels was performed according to the external standard method, integrating peak areas, acquired at 276 nm, at the retention time of the corresponding patulin standard. Four standard calibrated solutions of patulin at concentrations of 7.5, 12.5, 75 and 125 ng/ml were prepared. Sample extracts with patulin concentration higher than 125 ng/ml were appropriately diluted with acidified water and re-injected.

Molecular characterisation of *Penicillium* isolates

DNA extraction from pure cultures

Fungi were grown in Wickerham liquid medium (40 g glucose, 5 g peptone, 3 g yeast extract, 3 g malt extract in 1 l H_2O) incubated in darkness at 25±1 °C on an orbital shaker (150 rpm) for 2 days. DNA was extracted from 40 mg of filtered, frozen, and lyophilised mycelium by the EZNA Fungal DNA Miniprep Kit (Omega Bio-tek, Doraville, GA, USA) and recovered in sterile ultra-pure water.

Species-specific primers

DNA from collected *Penicillium* isolates was amplified according to Marek *et al.* (2003) using *P. expansum*-specific primers PEF (5'-ATCGGCTGCGGATTGAAAG-3') and PER (5'-AGTCACGGGTTTGGAGGGA-3'), that amplified a 404 base pairs (bp) fragment of the polygalacturonase gene.

Gene-sequencing

A fragment (approximately 450 bp) of *β*-tubulin gene was amplified using the primers Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2b ('5-ACCCTCAGTGTAGTGACCCT TGGC-3') according to Glass and Donaldson (1995). Sequencing reactions were performed by the Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems, Carlsbad, CA, USA) for both strands. Sequencing products were purified by gel filtration using Sephadex G-50 Superfine (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in Centri-Sep Spin Columns (Princeton Separations, Adelphia, NJ, USA) and analysed by a PRISM 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) for sequence generation. The two strands sequence assembling was performed by MT Navigator software (Applied Biosystems). The obtained nucleotide sequences were submitted to the online BLAST search engine of the NCBI to search for similarity of the query, and deposited in the European Nucleotide Archive (ENA, http://www.ebi.ac.uk/ena).

patN *gene analysis*

The presence of *patN* gene coding for the IDH enzyme in examined *Penicillium* strains was assessed by PCR amplification of its 600 bp region with specific primers ('5-CAATGTGTCGTACTGTGCCC-3') and (5'-ACCTTCAGTCGCTGTTCCTC-3'), according to Paterson *et al.* (2003).

Statistical analysis

Putative correlation between patulin production *in vitro* (μg/cm^2) and *in vivo* (μg/kg) was evaluated using the statistical software package Statistics for Windows (StatSoft, Tulsa, OK, USA).

3. Results and discussion

Isolates collection and determination of their toxigenicity

Fruits (11 pears, 9 yellow apples and 9 red apples) used for fungal isolation were singly homogenised and analysed for patulin content: 24 of the obtained purees proved to contain patulin at levels ranging from 5.3 to 10,109.1 μg/kg (Table 1). Six of the 11 pear samples were positive for patulin in the range 5.3-7,424 μg/kg. The highest contaminated sample proved to be P3, while P1, P2, P4, P10 and P11 were patulin-free. All the 9 yellow apple samples proved to be contaminated, showing patulin levels ranging from 7.4

Table 1. Patulin content (μg/kg of fresh weight) of purees made from apples and pears used for fungal isolation, and *in vitro* production (μg/cm^2 of plate surface) by the isolated *Penicillium* spp.

Host	*In vivo* content (μg/kg)	Fungal strain (ITEM ID)	*In vitro* production (μg/cm^2)	Accession numbers (β-tubulin)	*P. expansum* identity	*patN* gene
Pears						
P1	0	7005	1.77	LN896406	+	+
P2	0	7006	3.04	LN896407	+	+
P3	7,424	7007	2.79	LN896408	+	+
P4	0	7008	3.47	LN896409	+	+
P5	97.1	7009	1.56	LN896410	+	+
P6	190.7	7010	3.91	LN896411	+	+
P7	67.9	7011	1.45	LN896412	+	+
P8	5.3	7030	1.13	LN896431	+	+
P9	20.5	7031	0.04	LN896432	-	+
P10	0	7032	0.00	LN896433	-	+
P11	0	7033	0.00	LN896434	-	+
Mean	703.6		1.74			
Yellow apples						
YA1	10,109.1	7012	6.50	LN896413	+	+
YA2	7.4	7013	5.92	LN896414	+	+
YA3	227.9	7014	2.52	LN896415	+	+
YA4	305.6	7015	7,327.96	LN896416	+	+
YA5	116.6	7016	3.15	LN896417	+	+
YA6	2,448	7017	2.71	LN896418	+	+
YA7	3,411.2	7018	1.61	LN896419	+	+
YA8	1,010.4	7019	2.98	LN896420	+	+
YA9	300.9	7020	2.43	LN896421	+	+
Mean	1,993.0		817.31			
Red apples						
RA1	15.7	7021	2.94	LN896422	+	+
RA2	69.4	7022	3.12	LN896423	+	+
RA3	344.9	7023	4.48	LN896424	+	+
RA4	1,344	7024	1.24	LN896425	+	+
RA5	44.6	7025	1.28	LN896426	+	+
RA6	282.2	7026	1.85	LN896427	+	+
RA7	8,472	7027	1.86	LN896428	+	+
RA8	489.6	7028	1.13	LN896429	+	+
RA9	556	7029	2.01	LN896430	+	+
Mean	1,290.9		2.21			

to 10109.1 μg/kg. Also all the 9 analysed red apple purees contained patulin in the range 15.7-8,472 μg/kg. Mean patulin levels in purees obtained from pear, yellow and red apples were 709.6, 1,993.0 and 1,290.9 μg/kg, respectively. Similarly, Funes and Resnik (2009) found higher patulin levels in apple puree than in pear ones, with a 50% contamination (average of positive samples 123 μg/kg). These results are not surprising since parameters that affect patulin production, such as fruit pH, are often characteristic of a certain fruit and cultivar and thus the use of particular cultivars might influence patulin content in the final products (Morales *et al.*, 2008b). Furthermore, it has been reported that the profile of phenolic compounds, which are claimed to influence patulin accumulation (Sanzani *et al.*, 2009b), varies depending on cultivar (Eisele and Drakeb, 2005) and storage conditions (Napolitano *et al.*, 2004).

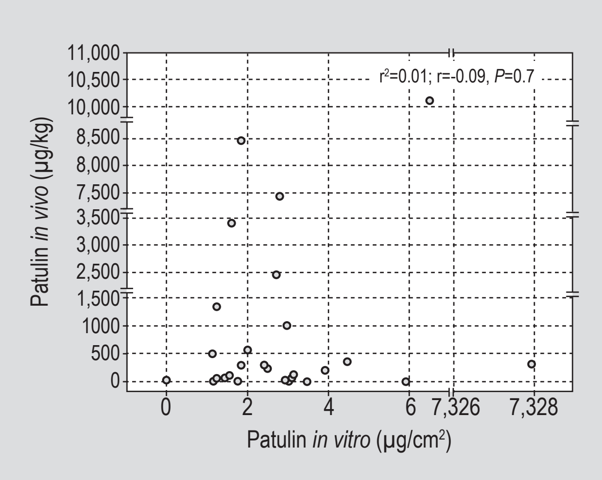

Figure 1. Correlation between patulin production *in vitro* (μg/cm^2) and *in vivo* (μg/kg).

The 29 isolates collected from each fruit and morphologically identified as *Penicillium* spp. were deposited in the ITEM fungal collection. Their patulin production potential was evaluated *in vitro*: detected patulin concentrations ranged from 0.04 to 7,327.96 μg/cm^2 (Table 1). Isolates originating from samples P1, P2, and P4, whose corresponding purees were free from patulin contamination, proved to produce the toxin *in vitro* (1.77, 3.04 and 3.47 μg/cm^2, respectively). Whereas isolates ITEM 7032 and 7033 originating from patulin free samples (P10 and P11) confirmed the absence of patulin production even *in vitro*. On average, the highest toxin amount was produced by strains isolated from yellow apples (817.31 μg/cm^2), followed by pears isolates (2.73 μg/cm^2) and red apple isolates (2.21 μg/cm^2). This different patulin production extent among isolates observed *in vitro* could be ascribed to a difference in the genetic producing potential among isolates, i.e. in the transcript level of one or more patulin biosynthesis genes.

These results suggest the absence of correlation between patulin accumulation in fruits and in corresponding fungal cultures (Figure 1). For instance, the strongest *in vitro* producer was ITEM 7015 (7,327.96 μg/cm^2 from YA4), whereas the most contaminate fruit was YA1 (10,109.1 μg/kg) from which ITEM 7012 originated. Similarly, Sommer *et al.* (1974) reported absence of correlation between the amount of patulin measured in fungal culture and patulin measured in corresponding host of fungal isolation.

In vivo, the presence of other patulin producing fungi might have contributed to toxin accumulation (Paterson, 2007). Indeed, Morales *et al.* (2008a) reported that highly contaminated packinghouse facilities might lead to an increase in the number of spores germinating in the same wound. Moreover, other *Penicillium* spp. that decay pome fruit, colonising wounds, might enhance the establishment of *P. expansum* infections (Sanderson and Spotts, 1995). Finally, it cannot be excluded that the collected isolates, although being the most prominent in growth, were not the strongest producers. On the contrary, the presence of unfavourable conditions for patulin production, such as the presence of antagonist microorganisms (i.e. yeasts or bacteria) might account for the lower patulin contamination of fruits from where strong patulin producers were isolated (e.g. YA4 and ITEM 7015). Indeed, bacteria (Ricelli *et al.*, 2007) and yeast (Spadaro *et al.*, 2008) that live saprophytically on pome fruit surface proved to be able to degrade patulin or prevent its production. Moreover, a prevalence of fungal primary metabolism in pears, thus explaining the lower patulin accumulation, and a higher secondary metabolism in apples postulated by Morales *et al.* (2008b) might account for the absence of patulin accumulation in samples P1, P2 and P4 from which the patulin producers ITEM 7005, 7006, and 7008 originated.

Molecular characterisation of *Penicillium* isolates

Once obtained these data, we proceeded with the characterisation of the isolates. However, the currently employed morphological and physiological techniques for *Penicillium* identification are time-consuming, labour-intensive, often inconclusive, and require mycological expertise (Sanzani *et al.*, 2014). Molecular diagnostic tools, rapid, specific, not subject to inter-observer variations, might be a reliable alternative. For instance, sequence analysis of β-tubulin (Glass and Donaldson, 1995), as well as the amplification with *P. expansum* species-specific primers (Marek *et al.*, 2003), are widely employed to facilitate the task. In our study, the species-specific amplicon (404 bp) was obtained for isolates from ITEM 7005 to ITEM 7030, therefore identified as *P. expansum*. These results were also confirmed by sequencing the β-tubulin gene. The isolates from ITEM 7005 to 7026, together with ITEM 7030, showed 100% similarity with the sequence of *P. expansum* strain CBS 325.48 (JQ965099.1), ITEM 7027 and ITEM 7028 showed 100% similarity with *P. expansum* strain CNU7002

(HQ225726.1), and ITEM 7029 showed 100% similarity with *P. expansum* MUT:1164 (KR709177.1). Concerning the three remaining isolates, ITEM 7031 showed 100% homology to both *P. camemberti* CBS 19067 (AY674369)/*P. commune* strain KACC 45904 (JF521510.1), ITEM 7032 and ITEM 7033 had 99% homology to *P. solitum* strain 49P (EU128541). The latter two did not produce patulin, consistently with information reported for this species (Frisvad *et al.*, 2007), whereas unexpectedly a low *in vitro* production was recorded for ITEM 7031. Patulin producing potential of collected isolates was further confirmed by PCR amplification using primers designed upon the *patN* gene, coding IDH. All of them resulted positive to the gene, although in two cases (ITEM 7032 and 7033) no patulin was detected (Table 1).

4. Conclusions

The early, rapid and specific detection of foodborne fungi is of paramount importance for ensuring both microbiological quality and safety of fruits and relevant derived products. However, apparently healthy pome fruit could be internally contaminated with toxigenic *Penicillium* species and very high levels of patulin. These fruit may enter the processing chain and affect the safety of derived products. It is therefore particularly important to check patulin presence and levels in the raw materials and during the processing chain, before the final packaging. Moreover, molecular detection tools DNA-based have to be carefully selected and applied for assessing toxigenic potential of a fungus, in fact the single evaluation of IDH gene presence might be not informative in this sense. Molecular taxonomy studies evidenced residual region of biosynthetic gene cluster in species closely related (Chang *et al.*, 2005; Susca *et al.*, 2014; Ward *et al.*, 2002), which can generate false positive results for potential toxin contamination, causing a misevaluation of the risk, specifically an overestimation.

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