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Development of a real-time PCR assay for *Penicillium expansum* quantification and patulin estimation in apples

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ABSTRACT

Due to the occurrence and spread of the fungal contaminants in food and the difficulties to remove their resulting mycotoxins, rapid and accurate methods are needed for early detection of these mycotoxigenic fungi. The polymerase chain reaction and the real time PCR have been widely used for this purpose. Apples are suitable substrates for fungal colonization mostly caused by *Penicillium expansum*, which produces the mycotoxin patulin during fruit infection. This study describes the development of a real-time PCR assay incorporating an internal amplification control (IAC) to specifically detect and quantify *P. expansum*. A specific primer pair was designed from the *patF* gene, involved in patulin biosynthesis. The selected primer set showed a high specificity for *P. expansum* and was successfully employed in a standardized real-time PCR for the direct quantification of this fungus in apples. Using the developed system, twenty eight apples were analyzed for their DNA content. Apples were also analyzed for patulin content by HPLC. Interestingly, a positive correlation ($R^2 = 0.701$) was found between *P. expansum* DNA content and patulin concentration. This work offers an alternative to conventional methods of patulin quantification and mycological detection of *P. expansum* and could be very useful for the screening of patulin in fruits through the application of industrial quality control.

1. Introduction

Unwanted fungal growth and mycotoxins contamination have become major problems in the Food chain (Negedu et al., 2011). Nowadays, the mycotoxins' topic has reached its paroxysm since these toxic molecules are being detected in the majority of food commodities. According to the Food and Agriculture Organization (FAO), approximately 25% of the world's food crops are annually contaminated with mycotoxins (Adams and Motarjemi, 1999; Negedu et al., 2011; Upadhaya et al., 2010). Among these well studied molecules, patulin is a toxic extrolite produced as a secondary metabolite by numerous species of filamentous fungi belonging to the genera *Penicillium*, *Aspergillus*, *Byssochlamys* and *Paecylomyces* (Moake et al., 2005; Puel, 2007). The genus *Penicillium* appears to be the major patulin producer, of which *Penicillium expansum* is by far the most worrisome species and the most commonly associated with patulin incidence (ElHariry et al., 2011; Morales et al., 2007). Although the presence of patulin is reported in various food commodities, its occurrence in pomaceous fruits, especially in apples and byproducts, remains the major concern (Baert et al., 2012; Salomão et al., 2009). Patulin has been revealed as acutely and chronically toxic causing genotoxic, cytotoxic, mutagenic as well as immunotoxic health effects (Puel et al., 2010). Due to its related hazards, the European Union has set maximum acceptable levels of 50, 25 and 10 μg patulin/Kg, respectively for fruit juices, nectars and fermented apple beverages, solid apple products and apple-based products for infants and young children (European Commission Regulation No 1425/2003 and No 1881/2006).

Lately, numerous surveys of patulin contamination levels in apples and derived products have been conducted in various countries around the world, including Turkey (Gökmen and Acar, 1998), Brazil

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(De Sylos, 1999; Iha and Sabino, 2008), South Africa (Leggott and Shephard, 2001), Belgium (Tangni et al., 2003), Iran (Majid Cheraghali et al., 2005), Italy (Spadaro et al., 2007), India (Saxena et al., 2008), Spain (Cano-Sancho et al., 2009; Murillo-Arbizu et al., 2009), Argentina (Funes and Resnik, 2009), Portugal (Barreira et al., 2010), China (Yuan et al., 2010), Saudi Arabia (Al-Hazmi, 2010), Tunisia (Zaied et al., 2013) and others. Most of these surveys were performed by a direct extraction of the toxin and revealed high percentages of contaminated samples, some of which have exceeded the tolerated patulin levels. As the other common mycotoxins, patulin is an appreciably stable compound, for which no credible removal process that guarantees a non-damaged product is present to date. Therefore, it is important to prevent its presence in the food chain by ensuring a fast and specific method to early detect the potential producing fungi before reaching the toxin's unacceptable level or even prior its synthesis. In this regard, there has been a recent wide-spread interest in molecular techniques such as the polymerase chain reaction (PCR) that ensures the fastest detection of fungal contamination in comparison with the conventional time consuming microbiological analysis. Several publications described the practical use of PCR in detecting mycotoxigenic fungi in food samples (Luque et al., 2011; Manonmani et al., 2005; Noorbakhsh et al., 2009; Shapira et al., 1996; Spadaro et al., 2011). With the continuous research improvement, the food sector has integrated the use of real time PCR (qPCR) and lately the multiplex real time PCR as new useful tools for detecting as well as quantifying the DNA of a specific or a broad-spectrum of mycotoxigenic species in a wide variety of food products (Hayat et al., 2012; Konietzny and Greiner, 2003; Rodríguez et al., 2012; Sardiñas et al., 2011; Selma et al., 2008; Vegi and Wolf-Hall, 2013). Regarding patulin, some conventional PCR assays have been reported for the detection of patulin-producing molds (Dombrink-Kurtzman, 2007; Paterson, 2004; Paterson et al., 2000). Recently, Rodríguez et al. (2011) reported the development of the first molecular method allowing the quantification of patulin-producing species in different food matrices by real-time PCR assay, using SYBR Green and TaqMan. However to our knowledge, no qPCR protocol has been elaborated yet to selectively detect and quantify the species with the highest economic impact on apples regarding patulin production, *P. expansum*.

In literature, the use of the qPCR technique was not only restricted to the quantification of the fungal contamination level in foodstuffs but a direct application of this method was also described to estimate the content of the associated mycotoxin level. In this regard, a number of papers have demonstrated the correlation between the amount of toxin and fungal DNA content (Atoui et al., 2007, 2012; Fredlund et al., 2008; Mulè et al., 2006; Schnerr et al., 2002). There are however no previous studies describing the development of such correlation to estimate the patulin levels in apples. Consequently, the objectives of the present work were to develop a quantitative real-time PCR assay to quantify the DNA amounts of the main patulin producer *P. expansum* in apple samples and to correlate *P. expansum* DNA with patulin content in apples in order to have an approximately estimation of the patulin contamination level.

2. Material and methods

2.1. Fungal strains

The fungal reference strains used throughout this study (Table 1) were obtained from the research unit TOXALIM of the National Institute for Agricultural Research (INRA), Toulouse, France. The isolates were maintained by regular subculturing on Potato Dextrose Agar (PDA) (Biolife, Milano, Italy) at 25 °C for 7 days and then stored as spore suspension in 15% glycerol at −80 °C.

Table 1
Fungal strains used in this study to test the specificity of the primer pair patF-F/patF-R.

Strain	Reference number
1 *Penicillium expansum*	NRRL 35694[a]
2 *Penicillium expansum*	NRRL 35695
3 *Penicillium griseofulvum*	NRRL 2159A
4 *Penicillium paneum*	M515[b]
5 *Penicillium carneum*	M521
6 *Penicillium chrysogenum*	NRRL 35692
7 *Penicillium glabrum*	NCPT 279[c]
8 *Penicillium nordicum*	NRRL 3711
9 *Penicillium vulpinum*	NRRL 2031
10 *Penicillium roqueforti*	P4 z88[d]
11 *Penicillium glandicola*	NRRL 2036
12 *Penicillium claviforme*	NCPT 39
13 *Penicillium brevicompactum*	NCPT 398
14 *Aspergillus clavatus*	NRRL 1980
15 *Aspergillus clavatus*	NRRL 1
16 *Aspergillus flavus*	NRRL 62477
17 *Aspergillus parasiticus*	NCPT 217
18 *Aspergillus fumigatus*	NRRL 35693
19 *Aspergillus niger*	NCPT 209
20 *Byssochlamys nivea*	NRRL 2615
21 *Byssochlamys nivea*	ATCC 24008[e]
22 *Byssochlamys fulva*	MUCL 14267[f]

[a] NRRL, Northern Regional Research Laboratory, Illinois, USA.
[b] Provided by M. Olsen, National Food Administration, Sweden.
[c] NCPT, New Collection Pharmacology-Toxicology Laboratory, INRA, France.
[d] Provided by J. Bauer, Technical University Munich.
[e] ATCC, American Type Culture Collection, Manassas, USA.
[f] MUCL, Mycothèque de l'Université Catholique de Louvain, Louvain, Belgium.

2.2. DNA isolations from pure fungal mycelia

All fungal strains listed in Table 1 were freshly cultivated on PDA media (Fluka, Saint-Quentin Fallavier, France) and allowed to grow for 7 days at 25 °C to enhance sporulation. The cultures were then used to inoculate with 10^5 spores a 250 ml Erlenmeyer flask containing 100 mL of Yeast extract Sucrose (YES) broth and incubated at room temperature under constant agitation for 4 days. Each liquid culture was then filtered through Whatman No. 42 filter paper and the mycelium was ground to fine powder under liquid nitrogen. Genomic DNA of the strains was obtained using the method described by Gardes and Bruns (1993) with some modifications. In this method, 200 mg of powdered mycelium was collected into microfuge tubes, homogenized with 700 µL of lysis buffer [2% CTAB (Amresco, Solon, USA), 1.4 M NaCl (Merck, Darmstadt, Germany), 20 mM EDTA pH 8 (Fisher Scientific, Illkirch, France), 100 mM Tris–HCl pH 8 (Biobasic Inc, Mundolsheim, France)] and incubated at 50 °C for 10 min then cooled on ice for 1 h. The sample was later treated with 500 µL of Phenol:Chloroform (50%/50%; v/v) (MP Biomedicals, Illkirch, France; Sigma–Aldrich, Saint-Quentin Fallavier, France), vortexed for 1 min and the supernatant was taken after centrifugation at 13,000 rpm for 15 min at 4 °C. The DNA was precipitated with an equal volume of ice-cold isopropanol (Amresco, Solon, USA) and incubated overnight at −20 °C. After incubation, the sample was again centrifuged at 13,000 rpm for 10 min at 4 °C. The pellet obtained was rinsed with 70% ethanol (Sigma–Aldrich, Saint-Quentin Fallavier, France), thoroughly air-dried and resuspended in 50 µL of sterile water. The DNA purity ratio and concentration were then measured using the Multiskan GO Microplate spectrophotometer (Thermo Scientific, Courtaboeuf, France).

2.3. Artificial contamination and incubation of apple samples

Twenty eight apples of different varieties (Fuji, Royal Gala and Golden Delicious), not showing any symptoms of fungal attack were randomly collected from the Lebanese markets. They were