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Short communication

Molecular characterization of patulin producing and non-producing *Penicillium* species in apples from Morocco

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ABSTRACT

The isolation of patulin-producing Penicillia in apples collected in different markets in four localities in Morocco is reported. Fungi were identified by β-tubulin sequencing and further characterized using a specific PCR-based method targeting the isoepoxydon dehydrogenase (IDH) gene to discriminate between patulin-producing and non-producing strains. Production of patulin was also evaluated using standard cultural and biochemical methods.

Results showed that 79.5% of contaminant fungi belonged to the genus *Penicillium* and that *Penicillium expansum* was the most isolated species (83.9%) followed by *Penicillium chrysogenum* (~9.7%) and *Penicillium crustosum* (~6.4%).

Molecular analysis revealed that 64.5% of the *Penicillium* species produced the expected IDH-amplicon denoting patulin production in these strains. However, patulin production was not chemically confirmed in all *P. expansum* strains. The isolation of IDH^-/$patulin^+$ strains poses the hypothesis that gentisylaldehyde is not a direct patulin precursor, supporting previous observations that highlighted the importance of the gentisyl alcohol in the production of this mycotoxin.

Total agreement between IDH-gene detection and cultural/chemical methods employed was observed in 58% of *P. expansum* strains and for 100% of the other species isolated.

Overall the data reported here showed a substantial genetic variability within *P. expansum* population from Morocco.

1. Introduction

Morocco is the third largest producer of apples in Africa after South Africa and Egypt and accounts for approximately 30% of African apple production (http://faostat.fao.org; Mounir et al., 2007). Post-harvest fungal pathogens cause substantial losses in the apple production in Morocco (Mounir et al., 2007) leading to significant economic losses for farmers and traders. *Botrytis cinerea* and *Penicillium expansum* are two well-known postharvest pathogens causing decay of apple fruits during storage (Calvo et al., 2007). In addition, *P. expansum* is also responsible for biosynthesis of patulin, a polyketide lactone mycotoxin that exhibits a number of toxic effects in humans and other animals (Doi and Uetsuka, 2014; Frizzell et al., 2014).

The multi-step synthesis of this toxin starts with the formation of 6-methylsalicylic acid and requires about ten enzymatic reactions even though, at present, the exact number and types of enzymes involved are still not well understood (Artigot et al., 2009; Puel et al., 2010; Tannous et al., 2014). The genes responsible for patulin biosynthesis in producing fungi are organized in clusters comprising 15 open reading frames, (Li et al., 2015) of which only 5 have been characterized to date, highlighting a large gap in knowledge of the genetic bases of its production (Tannous et al., 2014).

Patulin occurs mainly in apples that have been spoiled by mould growth, and therefore these fruits, including their processed products, are considered the most significant contributors of toxin in the diet (Murillo-Arbizu et al., 2009). Because of this, the levels of patulin in foods are regulated in many countries worldwide. In most cases, the maximum value has been set at 50 μg/l for fruit juice (Pires et al., 2012; Puel et al., 2010) even though some countries, such as those of the European Union, impose more stringent standards especially for infants and young children (Puel et al., 2010).

In Morocco, although several studies have been performed to describe the occurrence of different mycotoxins and toxigenic fungi in foods (El Adlouni et al., 2006; Juan et al., 2008; Roussos et al., 2006;

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Zinedine et al., 2007; Zinedine, 2010), little is known concerning the contamination of foodstuffs by fungi capable of producing patulin, especially those belonging to genus *Penicillium*. Therefore, in this study, we decided to investigate the occurrence of patulin producers in apples collected in different markets in four localities, Meknés, Midelt, Khenifra and Zaida in Morocco.

2. Materials and methods

2.1. Isolation and identification of fungal strains

Fungi were isolated from a total of 20 apples purchased in four Moroccan cities (5 apples for each city): Meknés, Midelt, Khenifra and Zaida. Briefly, all 20 apples were initially immersed in 2% (*w*/*v*) sodium hypochlorite solution for 1 min, rinsed 3 times in sterile distilled water and dried using sterile filter paper. From each sample, 5 pieces (~1 cm^2) were randomly selected and directly plated in Czapek Dox agar (CDA) (for 1 L of medium: Sucrose 30 g; $NaNO_3$ 2 g; K_2HPO_4 1 g; $MgSO_4$ 0.5 g; KCl 0.5 g; $FeSO_4$ 0.01 g; Agar 15 g; final pH 7.3 ± 0.2). Plates were incubated at 25 °C for 7 days.

The percentage of the infected parts was determined and the fungal colonies were counted, isolated, purified and maintained on CDA for further analysis.

Phenotypic identification of isolated fungi was performed by studying their morphology on CDA, malt extract agar (MEA) and Czapek yeast extract agar (CYA) after 7 days at 25 °C. Micromorphology was also examined using a light microscope (Leica DMR, Milan, Italy). *Penicillium* species were initially identified using phenotypic keys reported in Frisvad and Samson (2004).

2.2. Molecular characterization

Partial sequencing of the β-tubulin gene was made to confirm the identity of the fungal species isolated. Genomic DNA was extracted by using high-speed cell disruption method as described by Müller et al. (1998). In vitro amplifications were carried out in a total volume of 50 μl containing 25 μl of DreamTaq™ PCR master mix (Fermentas, Milan, Italy) plus 1 μl of genomic DNA template and 1 μl (10 mM) of each bt2a and bt2b primers (Glass and Donaldson, 1995).

Amplicons were purified using the QIAquik PCR purification kit (Qiagen, Milan, Italy) and sequenced at MWG-Eurofins, Ebersberg, Germany (www.eurofinsgenomics.eu) using the same primer set used for PCR.

BLAST searches, against GenBank database, were carried out for taxonomic recognition of our fungal strains using a threshold of ≥99% of similarity for species delineation.

A specific molecular method, based on amplification of isoepoxydon dehydrogenase (IDH) gene was employed to detect patulin producing moulds (Luque et al., 2011). Amplifications were performed according to Luque et al. (2011) with some modifications made for increasing confidence that absence of amplification in our isolates corresponded to true negative results. In brief, we set a multiplex PCR protocol by combining the IDH specific primers (FC2 and IDH2R) reported by Luque et al. (2011) with ITS1/ITS4 universal primers (White et al., 1990). In this way, all patulin producing strains generate two DNA bands (a specific 496 bp fragment from IDH gene and an amplicon of approximately 600 bp from rDNA) while non-producing strains produce only one band (~600 bp) relative to the internal positive control used. All experiments were repeated three times.

2.3. Patulin production and detection

Patulin production from our fungal isolates was determined using a reversed-phase, diode-array-detection, high-performance liquid chromatography (RP-DAD-HPLC).

Isolates were inoculated into 250 ml Erlenmeyer flasks containing 50 ml of Czapek's liquid medium (Sigma, Milan, Italy) supplemented with 5 g of yeast extract and incubated at 25 °C for 9 days in dark. The cultures were filtered under vacuum using Whatman n°4 filter paper and 15 ml of each filtrate were acidified to pH 2.0 with 100 μl of diluted HCl. Subsequently, 15 ml of chloroform was added and the lower phase was recovered in a flask and evaporated at 40 °C using a Rotavapor system. The filtrate was then eluted with 1 ml of methanol.

For patulin detection and identification, RP-DAD-HPLC analysis of the extracts was carried out with a Shimadzu system, consisting of a LC-10AD pump system, a vacuum degasser, a quaternary solvent mixing, a SPD-M10A diode array detector and a Rheodyne 7725i injector. Separation of each compound was performed on a 250 mm × 4.6 mm i.d., 5 μm Discovery C18 column, supplied by Supelco (Bellefonte, PA, USA), equipped with a 20 mm × 4.0 mm guard column. The column was placed in a column oven set at 30 °C. The injection loop was 20 μl, and the mobile phase was an isocratic combination of acetonitrile:H_2O (5:95; v:v) with a flow rate of 1.0 ml/min. UV–Vis spectra were recorded between 200 and 600 nm, and simultaneous detection by diode array was performed at 276 nm. The limits of detection was evaluated as three times the signal-to-noise ratio (LOD = 3S/N). The coefficient of variation (CV) for repeatability (n = 3) of retention time, peak height and peak area was within 1.0% respectively, and the CV for within-day repeatability (n = 3) and between-day precision (n = 10) of retention time was within 0.09% and 0.5%, respectively.

3. Results

A total of 39 fungal strains, belonging to three different genera, were isolated in this study (Table 1). *Penicillium* species were recovered from all examined samples of apples except those from Khenifra city (Table 1) and represented the most commonly isolated fungi (31/39; ~79.5%). The samples most contaminated by *Penicillium* spp. were those from Zaida (100%) followed by Midelt (80%) and Meknés (64.3%) (Table 1). Eight out of 39 strains were identified as *Aspergillus flavus* (6/39 strains) and *Alternaria alternata* (2/39 strains) representing the 15.4% and 5.1% respectively of the total fungi recovered (Table 1).

Among 31 *Penicillium* strains subjected to molecular identification via β-tubulin sequencing, the most part (26/31; ~83.9%) were identified as *P. expansum*, 3/31 (~9.7%) as *Penicillium chrysogenum* and 2/31 (~6.4%) as *Penicillium crustosum* (Table 2).

Molecular analysis, using a multiplex PCR-based method, revealed that 64.5% (20/31) of *Penicillium* species produced two expected DNA fragments: one of ~600 bp (rDNA internal positive control) and one of ~496 bp specific for the IDH gene (Fig. 1; Table 2), denoting patulin production in these strains (Luque et al., 2011). The remaining 11 *Penicillium* spp., although showing the rDNA amplicon, were IDH-negative and the absence of the specific IDH amplicon was also confirmed using FC2/IDH2R primers alone as described in Luque et al. (2011) (data not shown). Nevertheless, patulin production in all IDH positive *Penicillium* spp. was not confirmed by the standard chemical method employed in this study (Figs. 1 and 2; Table 2). In particular, RP-DAD-HPLC analysis showed that 58% (18/31) of the *Penicillium*

Table 1
Fungi recovered from apples from Meknés, Midelt, Khenifra and Zaida in Morocco.

Fungal species	Meknès*		Midelt*		Khenifra*		Zaida*	
	TC	%I	TC	%I	TC	%I	TC	%I
Aspergillus flavus	4	28.6%	1	10%	1	100%	0	0%
Penicillium spp.	9	64.3%	8	80%	0	0%	14	100%
Alternaria alternata	1	7.1%	1	10%	0	0%	0	0%
Total	14	100%	10	100%	1	100%	14	100%

* Results are from microbiological examination of 5 apples; TC = total count; %I = percentage of incidence.