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Original article

Mycoflora isolation and molecular characterization of *Aspergillus* and *Fusarium* species in Tunisian cereals

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ABSTRACT

Wheat, barley and maize are the mainly consumed cereals in Tunisia. This study aimed to determine the mycoflora of these cereals with special focus on the mycotoxigenic *Aspergillus* and *Fusarium* species. Freshly harvested samples and other stored samples of each type of cereal (31 and 34 samples, respectively) were collected in Tunisia and cultured for fungal isolation and identification. Identification of fungal genera was based on morphological *features. Aspergillus* and *Fusarium* species were identified by species specific PCR assays complemented with DNA sequencing. *Alternaria* (70.83%), *Eurotium* (62.50%), *Aspergillus* (54.17%) and *Penicillium* (41.67%) were the most frequent fungi isolated from wheat. *Penicillium* (75%), *Aspergillus* (70%), *Eurotium* (65%) and *Alternaria* (65%) were the most frequently recovered genera from barley. The predominant genera in maize were *Aspergillus* (76.19%), *Eurotium* (42.86%), and *Penicillium* (38.09%). *Aspergilllus*, *Penicillium*, *Fusarium* and *Alternaria* were detected in both stored and freshly harvested grain samples. The frequencies of contamination with *Aspergillus*, *Fusarium* and *Alternaria* were higher in freshly harvested samples, whereas *Penicillium* species were more frequent in stored samples. The predominant *Aspergillus* species detected were *A. flavus* and *A. niger*. The *Fusarium* species detected were *F. equiseti*, *F. verticillioides*, *F. nygamai*, and *F. oxysporum*. This study suggested the potential risk for Aflatoxins and, to a lesser extent, for Ochratoxin A in Tunisian cereals. This is the first survey about mycoflora associated with wheat, barley and maize in Tunisia.

1. Introduction

Foods and feeds, especially cereal grains, are susceptible to invasion by molds during pre harvesting, processing, transportation, or storage (Ellis et al., 1991). Fungal growth is one of the main causes of cereal spoilage. It not only generates great economic losses, but also can cause acute or chronic intoxication to human and animal, particularly through the synthesis of mycotoxins (Moss, 1996). The mainly fungal toxigenic genera are *Alternaria, Aspergillus, Fusarium* and *Penicillium* (Osweiler, 2000; Pittet, 1998). *Alternaria* and *Fusarium* species are often classified as field fungi, while *Aspergillus* and *Penicillium* species are considered as storage fungi, although these can grow if environmental conditions are favorable. The production of mycotoxins in commodities depends on both the environmental conditions and the toxigenic species present. Therefore, good harvesting and processing practices and early detection of mycotoxigenic species are key aspects in any strategy to prevent or reduce mycotoxins in foods. Precise and sensitive detection of the mycotoxigenic species is necessary since even very closely related species may produce a different array of mycotoxins (Sardiñas et al., 2011). The current methods being used for assessing mold presence are time-consuming, labor-intensive, and costly; require facilities and mycological expertise; and -above all- do not allow the identification of mycotoxigenic strains. DNA-based methods, in particular Polymerase Chain Reaction (PCR) based assays permit rapid, highly sensitive and specific detection of mycotoxigenic species in pure cultures

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and directly in food or raw samples. They have also been found to be a useful indicative of toxin contamination in samples (Gil-Serna et al., 2013; Jurado et al., 2006a; Sampietro et al., 2010).

Tunisia is a Mediterranean country, where climate is characterized by warm temperatures and relatively high and prolonged humidity, being favorable conditions for fungal growth in crops and commodities. The social and economic characteristics of the Tunisian population such as the food storage in house conditions can probably increase the mold growth and their secondary metabolite production. In Tunisia, cereals and derived products are the population's dietary. The consumption of wheat and barley cereals in the form of Couscous, Frik, Bsissa, pasta and traditional bread is a cultural tradition. Furthermore, maize is also one of the most important cereals since its use by Tunisian manufacturers to make maize oil used in cooking and frying and foods destined for infants and young children.

In Tunisia, there is no data on the mycoflora associated to the mainly consumed cereal, except two published surveys: the first determining the different groups of molds encountered in stored durum wheat and their changes during the storage period, in which *Alternaria*, *Fusarium*, *Penicillium* and *Aspergillus* were the most dominant post-harvest fungal genera isolated (Belkacem-Hanfi et al., 2013), the second characterizing the mycoflora of sorghum grains commercialized in the Tunisian retail market, where *Fusarium*, *Aspergillus* and *Alternaria* were the main genera isolated (Lahouar et al., 2015). Other reports were focused on *Fusarium* species related to the *Fusarium* Head Blight complex (FHB), one of the most important diseases in cereals, caused by *Fusarium* species and having severe impact on Tunisian wheat production (Fakhfakh et al., 2011; Gargouri-Kammoun et al., 2009). Recently, the contamination of Tunisian wheat, barley and maize samples with toxigenic *Aspergillus* species was studied. Results showed that wheat and barley are contaminated with only *A. flavus* and *A. niger* aggregate. As for maize, it contains in addition *A. parasiticus* but at low frequency (Jedidi et al., 2017).

In view of these considerations, the aim of this survey was to examine the fungal contamination of wheat, barley and maize consumed in Tunisia and to determine the most common fungal genera, using an integrated approach which combine traditional methods and DNA-based methods, namely species-specific PCR assays and sequencing of informative genomic regions (Jurado et al., 2006b; Sampietro et al., 2010).

2. Materials and methods

2.1. Cereal samples

A total of 65 samples, including wheat (n = 24), barley (n = 20) and maize (n = 21), were collected between February and June of 2011. At harvest time, 31 of those 65 samples (11 wheat, 10 barley and 10 maize) were randomly collected from fields located in different Tunisian cities (Sousse, Monastir, Mahdia, Sidi Bouzid, Kairouan and Beja) situated in three main regions (North, East and Center) where cereals are grown in Tunisia. The other 34 samples (13 wheat, 10 barley and 11 maize) were collected in storage facilities in three eastern cities (Sousse, Monastir and Mahdia) from different places such as the cereal office, some Tunisian houses and the retail market. All samples (each about 500 g) were stored in food polyethylene bags at 4 °C until being used in mycological analysis.

2.2. Analysis of the mycoflora

All the cereal samples were examined by the Direct Plating technique described by Pitt and Hocking (2009), to isolate their internal mycoflora: One hundred cereal grains per sample were surface disinfected in 2% active chlorine solution for one minute at room temperature. Then, they were rinsed twice in sterile distilled water for one minute and surface dried before direct plating. Plating was carried out in Petri plates (90 mm diameter, 10 grains/-plate) containing potato dextrose agar medium (PDA) (CONDA, Pronadisa, Madrid, Spain) and plates were incubated at 25 °C for seven days. The isolates obtained were sub-cultured on PDA, and the identification keys by Pitt and Hocking (2009) were used to determine their genera, and their section in the case of *Aspergillus* species (Section *Flavi* and *Nigri*). Monosporic cultures were done to *Aspergillus* and *Fusarium* colonies in order to obtain pure strains to be later identified by DNA based methods. Cultures were maintained on PDA at 4 °C and stored as spore suspensions in 15% glycerol at −80 °C.

The percentage of infected grains with a definite genus was calculated for each cereal sample, using the following formula:

Percentage of infected grains per sample (%)
= Number of infected grains by a genus in each sample
× 100/Total number of grains

Then, the relative frequency of contaminated samples (Fq) and the relative density (Rd) of the different isolated genera were calculated according to Marasas et al. (1988) as follows:

Fq (%) = Number of samples contaminated with a genus × 100/Total number of samples
Rd (%) = Number of isolates of a genus contaminating a sample × 100/Total number of isolates

Mean values were calculated to obtain average percentages of infected grains and average relative densities of the isolated genera in the different groups of samples. For these average values, the standard error, which represents the variability rate with respect to the mean, is also calculated as follows:

Standard error = standard deviation/
square-root of the number of samples

Data analyses were performed using STATGRAPHICS CENTURION XV.II (Statistical Graphics Corp., Herndon, VA).

2.3. Identification of the main Aspergillus and Fusarium toxigenic species by DNA-based methods

A total of 130 monosporic *Aspergillus* strains (84 *Aspergillus* section *Flavi*, 43 *Aspergillus* section *Nigri* and three other *Aspergillus* spp.) and 18 *Fusarium* strains were identified at species level using either species specific PCR assays or by sequencing a diagnostic genomic regions; These are a partial region of the Translation Elongation Factor-1 alpha (TEF-1α) gene for *Fusarium* strains and the Internal Transcribed Spacer (ITS) region of genomic rDNA for *Aspergillus* strain.

2.3.1. DNA extraction

Extraction of genomic DNA from fungal monosporic cultures was basically performed according to Querol et al. (1992) using three mycelial disks which were excised from the margin of a seven-day-old PDA plates and crushed against the wall of a 2 mL microcentrifuge tube, using a sterile pipette tip. The DNA concentration was estimated using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, NC, USA).

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