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Toxigenic molds in Tunisian and Egyptian sorghum for human consumption

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ABSTRACT

The objective of this study was to characterize the mycoflora of sorghum grains commercialized in the Tunisian retail market and to identify aflatoxins (AFs), ochratoxin A (OTA) and zearalenone (ZEA) producing species. Sixty four samples of sorghum (37 samples of Tunisian sorghum and 27 samples of Egyptian sorghum) were analyzed. Dilution plating (CFU, colony forming units) was used for fungal enumeration. The isolation of mycobiota was carried out by plating of grains on PDA and malachite green medium. *Aspergillus* section *Flavi* and section *Nigri* and *Fusarium* isolates were sub-cultured in CYA to test their ability to produce AFs, OTA and ZEA, respectively. The selected *Aspergillus* section *Flavi* and section *Nigri*, *Penicillium* and *Fusarium* isolates were subjected to specific PCR assays using published species-specific primers. The results revealed the dominance of *Fusarium* (95.3%), followed by *Aspergillus* (87.2%) and *Alternaria* (81.2%) species. The fungal counts ranged from 100 to $1.3 \cdot 10^4$ CFU/g for Tunisian sorghum and from 100 to $5.7 \cdot 10^3$ CFU/g for Egyptian sorghum. Among *Aspergillus* section *Flavi* isolates identified by molecular biology, *Aspergillus flavus* was the most dominant (90.1%) while *Aspergillus parasiticus* represent 9.9% only. About *Aspergillus* section *Nigri*, results showed the dominance of *Aspergillus niger* aggregate species, including *Aspergillus niger*, *Aspergillus tubingensis* and other species. Among *Fusarium* species, *Fusarium incarnatum* was the most dominant in both Tunisian and Egyptian sorghum. *Penicillium citrinum* was the dominant *Penicillium* species in the studied samples. More than 890 isolates belonging to the genus *Aspergillus* and *Fusarium* were tested in order to test their capacity to produce AFs, OTA and ZEA. The percentage of mycotoxin producing isolates in *Aspergillus* section *Flavi*, *A.* section *Nigri*, and *Fusarium* was 30.0%, 4.6% and 11.1%, respectively.

1. Introduction

Sorghum (*Sorghum bicolor* L.) is a cereal grain crop which originated in Africa, but is now mostly grown in Africa, Asia and America, primarily to ease food insecurity. According to ICRISAT/FAO (1996), as a global food source sorghum ranks the fifth after wheat, rice, corn, and barley and it is Africa's second most important crop in terms of tonnage. Sorghum is mostly grown in semiarid or subtropical regions due to its resistance to harsh weather conditions.

Sorghum grains are used as raw material for poultry, swine, and bovine feeds but are also intended for humans as a staple food in some African and Asian countries (Veiga, 1986). In fact, a large proportion of people in Africa, especially in the rural communities live on a diet primarily composed of staple foods prepared from cereals, including sorghum, tubers and plantains (Oniang'o et al., 2003). Sorghum is replacing maize as a staple food commodity in many rural settlements (Bandyopadhyay et al., 2007). Thus sorghum production in Africa is increasing significantly with a corresponding decline of rice and wheat production (FAOSTAT, 2010). North African and Tunisian populations consume cereals such as wheat, barley, corn and sorghum and cereal products. Moreover, cereals contribute to approximately 12% output and Tunisian households spend around 25% of their food expenditures on cereals. Indeed, cereals commercialized in Tunisia are imported and little is known about molds and mycotoxins contamination. Tunisia and other North African countries border the Mediterranean Sea, where climate is characterized by high temperature and humidity

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levels that may stimulate toxigenic mold growth and their secondary metabolite production.

Conversely, the overall world loss of foodstuff in the form of grains is considered to be 5% of the total production (FAO/WHO/UNEP, 1977). One of the main agents that cause these significant losses in cereals is fungi. In addition, these fungi are capable of producing not only losses in the organoleptic quality of thegrain but accumulating mycotoxins in cereals that can cause health problems to humans and animals (Chu and Li, 1994; Thiel et al., 1992). In Brazil, losses of 10–25% have estimated to occur throughout the trading process, partly due to contamination with toxigenic fungi and mycotoxins (Pedrosa and Dezen, 1991).

Alternaria, *Fusarium*, *Cladosporium*, *Curvularia*, *Phoma*, *Aspergillus*, and *Penicillium* are genera associated with the contamination of sorghum grains (Pitt et al., 1994; Gonzalez et al., 1997; Da Silva et al., 2000; Alves dos Reis et al., 2010). These fungi have different requirements of humidity and temperature. Thus depending on these factors fungi are able to contaminate grains from harvest through transport and storage. In addition, if the conditions in the grain are favorable they can grow and produce mycotoxins. Therefore prevention of fungal growth effectively conduces to prevention of mycotoxin accumulation (Barros et al., 2008). A number of fungal species associated with sorghum, belonging mainly to the genera *Fusarium* (*Fusarium verticillioides*, *Fusarium graminearum*, *Fusarium equiseti*), *Alternaria* (*Alternaria alternata*), *Aspergillus* (*Aspergillus flavus*), and *Penicillium* (*Penicillium funiculosum*) have been reported to produce mycotoxins and contaminate sorghum (Alves dos Reis et al., 2010; Da Silva et al., 2006; Isakeit et al., 2008).

The growing importance of sorghum as food and feed in a high number of countries and the possibility that this grain is contaminated with mycotoxin shasled the Codex Alimentarius Commission to ask for studies to obtain more data on the occurrence of mycotoxins in sorghum. The main objective is to try to minimize the problem of the mycotoxins in sorghum around the world (Codex Alimentarius Commission, 2012).

In view of these considerations, the aim of the present study was to assess the contamination level in sorghum samples intended for human consumption from the retail market of Tunisia. For this purpose the mycobiota present in the samples was first analyzed and subsequently the ability of a number of fungal isolates belonging to *Aspergillus*, *Penicillium* and *Fusarium* to produce AFs, OTA and ZEN was tested under optimal conditions.

2. Materials and methods

2.1. Samples

In 2011–2012, a total of 64 sorghum samples were collected in the retail market of Sousse, and Monastir, two regions of the center of Tunisia characterized by coastal and humid climate. Thirty seven (37) samples of sorghum were Tunisian products while twenty seven (27) were imported from Egypt. The samples (each about 500 g) were kept in food polyethylene bags at 4 °C.

2.2. Mycobiota determination

The study of the mycobiota present in the samples under observation was carried out by quantitative enumeration (fungal counts) and determination of internal fungi.

2.2.1. Fungal counts

The quantitative enumeration of fungal propagules was done on solid media using the surface-spread method. Serial dilutions in peptone saline water were made and 100 µl aliquots were inoculated onto plates of potato dextrose agar (PDA) supplemented with 100 ppm of chloramphenicol and plates of dichloran rose Bengal chloramphenicol (DRBC) agar (5.0 g of peptone, 10 g of dextrose, 1.0 g of monopotassium phosphate, 0.5 g of magnesium sulfate, 0.02 g of 2-6-dichloro-4-nitro-aniline, 0.025 g of rose Bengal, 0.10 g of chloramphenicol and 15 g of agar in 1 L of distilled water). Plates were incubated at 25 °C for 7 days. Dilution plates with 10–100 CFU were used for enumeration and the results were expressed as CFU per gram of sample. However, in samples with a low level of fungal contamination, plates with less than 10 CFUCFU at the lowest tested dilution (10^{-1}) were recorded. The molds present on the plates were identified to genus level using the identification keys by Pitt and Hocking (1997). *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. were isolates were kept in potato dextrose agar plates.

2.2.2. Internal mycobiota

For isolation of the internal mycobiota, a subsample of 200 kernels of each sample was surface disinfected in a 2% aqueous solution of sodium hypochlorite for 2 min, and rinsed twice with sterile distilled water. The kernels were aseptically plated in dichloran rose Bengal chloramphenicol (DRBC) agar (5 kernels/plate). Plates were incubated at 25 °C for 7 days. After incubation, they were examined for fungal growth, and the molds present on the kernels were identified to genus level using the methods of Pitt and Hocking (1997). Moreover, malachite green agar (11.25 g of peptone, 0.75 g of KH_2PO_4, 0.375 g of $MgSO_4-7H_2O$, 0.0019 g of green malachite, 0.075 g of chloramphenicol, and 15 g of agar in 750 mL of distilled water) was used for plating in order to detect *Fusarium* species. *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. were transferred to potato dextrose agar plates. Results in DRBC agar were reported as percentage of infected kernels. The isolation frequency (Fq) and the relative density (Rd) of genera and species were calculated according to Marasas et al. (1988) as follows:

$$\text{frequency } (\%) = \frac{\text{Number of samples of occurrence of a genus}}{\text{Total number of samples}} \times 100$$

$$\text{relative density } (\%) = \frac{\text{Number of isolates of a genus or species}}{\text{Total number of fungi or genus isolates}} \times 100$$

2.2.3. Molecular characterization of the isolates

2.2.3.1. A- DNA extraction. Selected fungal strains were cultured in 500 µl of Malt Extract broth (malt extract: 20 g, peptone: 1 g, glucose: 20 g, distilled water: 1000 ml). After 2 days of growth at 28 °C, mycelium was centrifuged (10 min–17,500 × g)and DNA was extracted with 300 µl of DNA extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% w/v SDS). The mycelium suspension was lysed by vortexing with five 2.8 mm stainless steel beads (Precellys, BertinTechnologies, France) during 10 min. After centrifugation at 17,500 × g for 10 min, 150 µl of 3 M sodium acetate were added to the supernatant. Then, supernatant was stored at −20 °C for 10 min and centrifuged (10 min–17,500 × g). The DNA-containing supernatant was transferred to a new tube and nucleic acids were precipitated by adding 1 volume of isopropyl alcohol. After 5 min of incubation at room temperature, the DNA suspension was centrifuged and the DNA pellet was washed with 70% ethanol to remove residual salts. Finally, the DNA was