Toxigenic molds in Tunisian and Egiptian 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 *Aspergillu*s 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 10^4 CFU/g for Tunisian sorghum and from 100 to 5.7 10^3 CFU/g for Egyptian sorghum. Among *Aspergillus* section *Flavi* isolates identified by molecular biology, *Aspergillusflavus* was the most dominant (90.1%) while *Aspergillusparasiticus* represent 9.9% only. About *Aspergillus* section *Nigri*, results showed the dominance of *Aspergillusniger* aggregate species, including *Aspergillusniger*, *Aspergillustubingensis* and other species. Among *Fusarium* species, *Fusariumincarnatum* was the most dominant in both Tunisian and Egyptian sorghum. *Penicilliumcitrinum* 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.

Keywords: mycobiota, sorghum, *Fusarium*, *Aspergillus*, mycotoxin.

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 communitieslive 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 levelsthat 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 ofthemainagents thatcausethesesignificant lossesin cerealsisfungi. In addition, thesefungiarecapableof producingnotonlylosses in theorganolepticqualityof thegrainbutaccumulatingmycotoxins incerealsthatcan causehealthproblemstohumans and animals (Chuand Li, 1994;Thiel et al., 1992;). In Brazil, losses of 10-25% haveestimated to occurthroughoutthetradingprocess, partlydue to contaminationwithtoxigenicfungiandmycotoxins(Pedrosa andDezen, 1991).

Alternaria, Fusarium, Cladosporium, Curvularia, Phoma, *Aspergillus*, and*Penicillium*are genera associatedwiththecontamination of sorghumgrains (Pitt et al., 1994; Gonzalez et al., 1997; Silva et al., 2000, Alves dos Reis et al., 2010). Thesefungihavedifferentrequirements of humidityand temperature. Thusdepending onthese factors fungiareable to contaminategrainsfromharvestthrough transport andstorage. In addition, iftheconditions in thegrainare favorablethey can growandproducemycotoxins. Therefore prevention of fungal growth effectively conduces to prevention of mycotoxin accumulation (Barros et al., 2008).A number of fungalspeciesassociatedwithsorghum, belongingmainly to the genera *Fusarium* (*F. verticillioides, F. graminearum, F. equiseti*), *Alternaria* (*A.alternata*), *Aspergillus* (*A. flavus*), and*Penicillium*(*P.funiculosum*) havebeenreported to producemycotoxinsandcontaminatesorghum(Alves dos Reis et al., 2010; Da Silva et al., 2006; Isakeit et al., 2008).

Thegrowingimportanceof sorghumas foodandfeedin a highnumberof countries andthepossibilitythatthisgrainis contaminatedwithmycotoxinshasledtheCodexAlimentariusCommissionto ask forstudies toobtainmoredata ontheoccurrence of mycotoxins in sorghum. Themainobjective is to try to minimizetheproblem of themycotoxins in sorghumaroundthe world (CodexAlimentariusCommission, 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 was 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 4ºC.

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 ofdichloran 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 25ºC 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 CFU at the lowest tested dilution (10^{-1}) were recorded. The molds present on the plates were identified to genus level using the identification keys byPitt 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 minutes, 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 25ºC 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:

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

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

2.2.3-Molecular characterization of the isolates

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- 17500 x g)and DNA was extracted with 300 µl of DNA extraction buffer (200 mMTris-HCl, pH 8.5, 250 mMNaCl, 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 17500 x 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- 17500 x 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 resuspended in 50 µl of TE buffer. The quality and concentration of DNA were determined in a NanoDropND-1000 spectrophotometer (NanoDrop Technologies, Wilmington USA).

b- PCR procedure

Identification of *Aspergillus* section *Flavi* and *Nigri* species was done by molecular characterization using specific primers, whereas identification of *Fusarium* isolates was conducted by partial sequencing of the Transcription Elongation Factor 1 alpha (TEF-1α) gene and the Beta Tubulin (β-tubulin) gene using the primer pairs BT2A/BT2B and EF-1/EF-2, respectively. Additionally, the identification of other potential mycotoxin producer isolates was carried out by partial sequencing of the ITS region and the β-tubulin gene (Table 1).

To identify *Aspergillus* section *Nigri*, a multiplex PCR with the primer pairs for *A. niger/A. awamori*, *A. carbonarius* and *A. tubingensis* detailed in Table 1, was conducted. PCR reactions were performed in a final volume of 10µL containing 1 µL of buffer 10x, 0.25 µL of dNTPs (10 mM), 0.5 µL of each primer (10 mM), 0.1 µL of DNA polymerase (5 U/µL) (DFS-Taq DNA polymerase, BIORON, Germany), 4.65 µL of water and 1 µL of DNA sample (10 ng/µL).

The identification of *A. flavus* and *A. parasiticus* was carried out using AfAflT-F, AfAflT-R. Two DNA extracts of *A. flavus* CECT 2695 and *A. parasiticus* CECT 2681 were used as reference. PCR reactions were performed in a final volume of 10µL, containing 1µL of buffer 10x, 0.25µL ofdNTPs (10 mM), 0.5µL of each primer (10 µM), 0.1 µL of DNA polymerase (5 U/µL) (DFS-Taq DNA polymerase, BIORON, Germany), 5.65 µL of water and 1 µL of DNA sample (10 ng/µL).

For *Fusarium*identification, partial sequencing of the TEF-1α and β-tubulin genes was conducted in PCR reactions in a final volume of 50 µL with the same cocktail proportions. PCR products were cleaned with the Ultra Clean PCR Cleanup DNA Purification kit (MOBIO, USA). The PCR purified products were directly sequenced by the company Macrogen (Korea). Sequence similarity searches were performed with BLAST(basic local alignment search tool) against public databases.

2.3- Mycotoxin-producing ability

2.3.1- OTA-producing ability

The isolates belonging to recognized OTA-producing species of*Aspergillus* and *Penicillium* genus were evaluated using a previously described high performance liquid chromatography (HPLC) screening method (Bragulat et al., 2001). Briefly, the isolates were grown on plates containing Czapek yeast extract agar (CYA) (Pitt and Hocking, 1997) and

incubated at 25ºC for 7 days. From each culture, three agar plugs were removed from different points of the colony and extracted with 1 mLof methanol. The obtained extracts were filtered and analyzed by HPLC. OTA was quantified on the basis of the HPLC fluorometric response compared with that of the standard OTA (Sigma Chemical Co., St. Louis, MO). The detection limit for OTA is 0.21µg/kg (Bragulat et al., 2001).

2.3.2- Aflatoxin and zearalenone -producing ability

The isolates belonging to *Aspergillus* section *Flavi* and *Fusarium* spp. were evaluated for aflatoxin and zearalenone-producing ability, respectively. The modified Bragulat method described previously for ochratoxin A was used for both mycotoxins. The only difference was that for aflatoxin-producing ability the coconut agar medium (CAM) (Lin and Dianase, 1976) was also employed. The obtained extracts were filtered and analyzed by HPLC. The mycotoxins were quantified on the basis of the HPLC fluorometric response compared with that of the standards ofaflatoxins and zearalenone (Sigma Chemical Co., St. Louis, MO). An ultraviolet (UV) light photochemical derivatizator (LC Tech, Dorfen, Germany), set at 254 nm, and located between the column and detector,was used for aflatoxins quantification.The detection limit for aflatoxinswas 0.60 µg/kg and for zearalenone0.017 µg/kg.

3.Results and Discussion

3.1-Mycobiota determination

Fungal enumeration results associated with sorghum grain purchased at the retail market in Tunisia during 2011 and 2012 are presented in table 2. The studied samples were from Tunisia and Egypt. The mean fungal count value of the total samples was 2.5 10^3 CFU/g (100 - 1.3 10^4 CFU/g for Tunisian sorghum and 100 - 5.7 10^3 CFU/g for Egyptian sorghum). We did not find significant differences between both groups of samples. The fungal levels were lower than those obtained by Lefeyedi et al. (2005) with counts of 2.7 10^4 CFU/g. The

commonly isolated fungi were a species of *Aspergillus, Fusarium, Rhizopus, Alternaria* and *Penicillium*. *Aspergillus*species belonging to the sections *Flavi* and *Nigri* predominated.

The internal mycobiota associated with sorghum samples is shown in Table 3. The presence of fungi was observed in all of the sorghum samples analyzed (37 from Tunisia and 27 from Egypt). The main genera isolated, listed in decreasing order of frequency were *Fusarium* (95.3%), *Aspergillus* (87.5%), *Alternaria* (81.2%), *Curvularia* (78.1%), *Drechslera*(78.1%), *Penicillium* (64.0%), *Eurotium* (43.7%), and *Cladosporium* (32.8%). Regarding the relative density, the three more important genera were *Fusarium* (23.6%), *Alternaria* (17.3%) and *Aspergillus*(12.2%). The predominance of these filamentous fungi found in this work agrees with other studies of sorghum mycobiota in Brazil (da Silva et al., 2000), Thailand (Pitt et al., 1994), and India (Sharma et al., 2011). The high presence of storage molds like *Aspergillus*, *Penicillium* and *Eurotium* was due to the fact that the samples were not freshly harvested.

Regarding genus *Aspergillus* the two main sections are *Nigri* and *Flavi* with a relative density of 7.0% and 5.2%. The low frequency of isolation of *A. flavus* and *A. parasiticus* from sorghum grains is in agreement with the study of Ratnavathi and Sashifhar (2003) and the study of Alves dos Reis et al. (2010). According to those authors, the physical characteristics and biochemical composition of the grains make sorghum less susceptible than other cereals to infection with *Aspergillus*. However, da Silva et al. (2000) showed the presence of *Aspergillus* genus in Brazilian sorghum with high frequency (42.7%), and *A. flavus* was the prevalent specie, although they did not surface-disinfected the grains before plating.

In our samples, *A. flavus* was, as well, the prevalent species from *Aspergillus* section *Flavi* in Tunisian and Egyptian sorghum grains (Table 4). We obtained 91 isolates from section *Flavi*, and 82 isolates (90.11%) were identified as *A. flavus*. Among 347 isolates of *Aspergillus* section *Nigri*, *A. niger* aggregate other than *A. niger/A.awamori* and *A. tubingensis* were the most dominant. *A. niger* clade includes 10 biseriate species, e.g. *A. brasiliensis, A. acidus, A.*

neoniger. However, only *A. Niger* is known to be able to produce OTA (Varga et al., 2011) (Table 4). Other *A. niger* aggregate species in Egyptian sorghum were more common than *A. niger* and *A.tubingensis,* while *A. niger* was the most dominant in Tunisian sorghum. These species were dominant in sorghum from Argentina, Thailand and Brazil (Alves dos Reis et al., 2010; Gonzalez et al., 1997; Pitt et al., 1994). *A. carbonarius* was not detected in the present study; however, this species showed a higher distribution in Indian sorghum samples (Priyanka et al., 2014).

Conversely, *Aspergillus* sections *Circumdati* and *Terrei* have been detected in sorghum seeds, being *Aspergillus flocculosus* and *A. terreus* the species isolated in this study, respectively. *Eurotium repens* and *Eurotium chevalieri* were the most predominant *Eurotium* species in the analyzed sorghum samples. Other species identified were *Aspergillus versicolor, A. sidowii* and *Emericella nidulans.*

Contamination of sorghum grains with the genus *Penicillium* was very low. Among the 30 *Penicillium* spp. isolates, isolated as internal mycobiota from Tunisian and Egyptian sorghum grain, the predominant species recorded was *P. Citrinum*(20 isolates). Other species found in the study were *P. Funiculosum* (6 isolates) and *P. Purpurogenum* (4 isolates). Similar results were obtained by Gonzalez et al. (1997) in Argentinian sorghum. None of the *Penicillium* species found has been cited as OTA producer species.

Isolates of *Fusarium* were the most prevalent components of the internal seedborne mycobiota of sorghum grains for the two groups of samples. Four hundred and fifty one isolates of *Fusarium* were inoculated on potato dextrose agar (PDA) plates. Morphological/cultural characters of these isolates were studied on PDA. From these, 59 isolates from different locations representing different morphological/cultural characters isolates were selected for full identification.

The identification of the species corresponding to the 59 *Fusarium* isolates was carried out using Blast n-searches in the Gen bank by determination of level of identifying with known *Fusarium* sequences. Twelve species of *Fusarium- F. incarnatum* (*F. equiseti* complex) (37 isolates*), F. Verticillioides* (*Gibberella moniliformis*) (4 isolates), *F. Thapsinum* (2 isolates), *F. Proliferatum* (2 isolates), *F. Pseudonygamai* (2 isolates), *F. Nelsonii* (3 isolates), *F. Venenatum* (2 isolates), *F. Pseudograminearum* (1 isolate), *F. Brachygibbosum* (1 isolate), *F. Acuminatum* (1 isolate), *F. Lacertarum* (1 isolate), *F. sambucinum* (*Gibberella pulicularis*) (2 isolates) and *G. xylarioides* (1 isolate) were identified based on sequence similarity. *Fusarium incarnatum* was identified as a predominant (37 of the 59 isolates tested) species in *Fusarium* sorghum mold complex. This species has been associated with sorghum seeds in India (Sharma et al., 2011; Divakara et al. 2013) and Argentina (Gonzalez et al., 1997). The species of the *Liseola* section (*F. verticillioides*, *F. thapsinum*, *F. proliferatum* and *F.pseudonygamai*) with 10 isolates are also important. *Fusarium* species included in the section *Liseola* have been reported to be associated with sorghum grain mold complex in different countries (Divapara et al., 2013). In fact, *F. thapsinum* is the predominant species in India (Sharma et al., 2011) and Uganda (Prom et al., 2011), and has been reported to be common in sorghum grain (Leslie et al., 2005). In addition, species of this section have the capacity to produce fumonisins, moniliformin, fusaric acid, beuvericin (Leslie et al., 2005). The incidence of *Fusarium* species from *Liseola* section should be responsible of the fumonisin contamination in sorghum from different countries (Scott, 2012).

Alternaria spp. also showed high isolation frequency in the studied samples and *A. tenuis* was the main specie isolated in both groups of sorghum samples (58.28%). Other species identified were *Curvularia lunata, Curvularia pallescens, Phoma sorghina, Cladosporium herbarum, Cladosporium fulvum* (syn. *Passolorafulva*),*Chaetomium funicola, Bipolaris*spp., *Ulocladium* spp.,*Epicoccum nigrum, Trichothecium roseum, Nigrospora*

oryzae, Rhizopus oryzae,Mucor spp.. These species have been found by other researchers in sorghum samples from other countries like Argentina, Bangladesh, Brazil, South Africa and Thailand (Alves dos Reis et al., 2010; Gonzalez et al., 1997; Isakeit et al., 2008; Lefyedi et al., 2005; Pitt et al., 1994).

Correct species identification is necessary for evaluating the possible risk of mycotoxins contamination as not all the species within a genus are able to produce mycotoxins and the production level varies among mycotoxin-producing species. Over the past decades, the classical fungal taxonomy has shifted to molecular identification because it is very difficult to establish systems of taxonomy based on phenotypic characteristics(Begerow et al., 2010). Therefore, the importance of molecular methods to differentiate taxa for important mycotoxin- producing fungi such as *Aspergillus* spp. *Penicillium* spp. and *Fusarium* spp. is emphasized. The sequences most commonly used to distinguish among species are portions of genomic sequences encoding nuclear ribosomal genes which include the large subunit (26S or 28S) and the small subunit (18S) separated by the Internal Transcribed Space (ITS), the β-tubulin gene and the transcription elongation factor 1 alpha gene (Glass and Donaldson, 1995; O'Donnell, 1993; White et al., 1990). Not all primers used to amplify the regions mentioned above work equally well for all species, so, in each case we have to use the most widely accepted marker for differentiation among representatives of each genus or section.

Nevertheless, little work has been done to characterize fungal diversity in sorghum by molecular identification. Da Silva et al. (2006) studied the genetic variability of 21 *Fusarium verticilloides* strains isolated from sorghum cultivated in the State of São Paulo, Brazil, using SPAR (Single Primer Amplification Reaction) markers while Divakara et al. (2013) identified and characterized 27 *Fusarium* spp., isolated from 58 sorghum seed samples collected across India, sequencing the transcription elongation factor 1 alpha gene and analyzing the Inter

Simple Sequence Repeat (ISSR) polymorphisms. Additionally, Priyanka et al. (2014) studied the diverse distribution of 200 *Aspergillus* strains from a total of 320 samples of maize, paddy, sorghum and groundnut by species-specific PCR assays. However, to our knowledge, there are no previous publications about the mycobiota of African sorghum in which molecular methods are used to differentiate taxa. In this study, not only *Fusarium* and *Aspergillus* isolates but also *Penicillium* and other interesting isolates have been studied by molecular methods. This, together with the high number of isolates identified (more than 600 isolates) is crucially important to clarify the occurrence of mycotoxin- producing strains in sorghum samples from Africa.

3.2- Mycotoxin-producing ability

In *Aspergillus* section *Flavi,* 33 over 91 tested isolates (36.26%) were able to produce aflatoxins. All positive isolates belonged to *Aspergillus flavus* species (40.24%) and produced detectable levels of aflatoxins B1 and B2 at concentrations ranging from 0.60 to 15.00µg/kg. Similar studies were carried out in sorghum from Brazil and India. In Brazilian sorghum, Da Silva et al. (2000) found 64.4% aflatoxigenic *A. flavus* strains, aflatoxin B1+B2 ranging from 12.0 to 3282.5 µg/kg. In Indian sorghum samples, Divakara et al. (2014) found 83.8% aflatoxigenic *A. flavus* strains.

Regarding *Aspergillus* section *Nigri,* we tested three hundred forty-seven (347) isolates, the percentage of OTA positive isolates was very low(4.61%).This low percentage is related to the absence of detecting isolates of *Aspergillus carbonarius* which is the species in the *Nigri* section with the highest percentage of OTA producing isolates.The 16 ochratoxigenic isolates produced OTA at concentrations ranging from 0.21 to 1.50µg/kg. One isolate of *Aspergillus flocculosus* (*Aspergillus* section *Circumdati*) showed the highest capacity to produce OTA. The isolate produced 492.33µg/kg. This species has been reported

to produce large amounts of OTA by Frisvad et al. (2004), but had not been isolated from sorghum before.

Finally, we found 50 isolates of *Fusarium* with capacity to produce zearalenone (11.09%). These isolates produced detectable levels of this mycotoxin at concentrations ranging from 0.017 to 4.61µg/kg. These low values could be explained by the predominance of species of *Fusarium equiseti* complex and no detection of species such as *F. graminearum* and *F. culmorum* that are the higher zearalenone producers.

This study is the first report about the incidence and mycotoxigenic capacity of the species belonging to the three main toxigenic genus (*Aspergillus*, *Penicillium* and *Fusarium*) with capacity to contaminate sorghum destined for human consumption.

4. Conclusion

The results revealed the dominance of *Fusarium* species followed by *Aspergillus* species and *Alternaria* in the studied samples. No differences in mycobiota were observed between samples from Tunisia and Egypt. The results presented in the study show the need for routine monitoring of sorghum during both harvest and storage in order to prevent from fungal growth and mycotoxin accumulation in the sorghum grains, as well as the need for routine analysis of sorghum batches for mycotoxins prior further processing for food production.

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Table1. Primers used for PCR identification

Fungal species	Primer pairs	T[a] annealing	Forward	Reverse	References
A. niger	NIG1, NIG2	60°C	5'-GATTTCGACAGCATTT(CT/TC)CAGAA-3'	5'-AAAGTCAATCACAATCCAGCCC-3'	Susca et al., 2007
A. carbonarius	AckS10R, AckS10L	60°C	5'-CCCTGATCCTCGTATGATAGCG-3'	5'-CCGGCCTTAGATTTCTCTCACC-3'	Selma *et al.*, 2008
A. tubingensis	TUB1, TUB2	60°C	5'-TCGACAGCTATTTCCCCCTT-3'	5'-TAGCATGTCATATCACGGGCAT-3'	Susca *et al.*, 2007
A. Flavus, A. parasiticus	AfAflT-F, AfAflT-R	70°C	5'-CGCGCGAGATACTTCTTATACT-3'	5'-GAGCCCACTTCGAAAATACC-3'	Godet and Munaut, 2010
Fusarium spp.	BT2A, BT2B	60°C	5'-GGTAACCAAATCGGTGCTGCTTTC-3'	5'-ACCCTCAGTGTAGTGACCCTTGGC-3'	Glass and Donaldson, 1995
	ef1, ef2	53°C	5'-ATGGGTAAGGARGACAAGAC-3'	5'-GGARGTACCAGTSATCATGTT-3'	O'Donnell *et al.*, 1998
Other unidentified isolates	ITS1, ITS4	56°C	5'-TTTCCGTAGGTGAACCTGC-3'	5'-TCCTCCGCTTATTGATATGC-3'	

Table2. Mean values of total mold counts, *Aspergillus*, *Fusarium*, *Penicillium*, and *Rhizopus* counts in sorghum samples purchased at Tunisian retail market during 2011 and 2012.

Mean value	Sorghum samples counts (CFU/g)		
	Tunisia	Egypt	Total samples
Total mold counts	$2.8\ 10^3$	$2.0\ 10^3$	$2.5\ 10^3$
Aspergillus section *Flavi*	$1.6\ 10^2$	$3.2\ 10^2$	$2.8\ 10^2$
Aspergillus section *Nigri*	$4.8\ 10^2$	$4.2\ 10^2$	$4.4\ 10^2$
Penicillium	2	$1.0\ 10^2$	$0.6\ 10^2$
Fusarium	$1.1\ 10^2$	$4.4\ 10^2$	$2.2\ 10^2$
Rhizopus	$0.2\ 10^2$	$0.8\ 10^2$	$0.5\ 10^2$

Table3. Internal mycobiota of sorghum purchased at Tunisian retail market during 2011 and 2012 (mean and mean standard error).

Fungus	Incidence (percentage of infected samples)[1]			Relative density (percentage of isolated genus)[2]			Average percent of infected grains[3]		
	Tunisia	Egypt	total	Tunisia	Egypt	total	Tunisia	Egypt	total
Aspergillus	86.11±2.61	89.28±0.57	87.50±1.49	12.84±1.71	11.45±1.72	12.22±1.21	6.15±1.74	2.96±0.38	4.73±0.99
Section *Flavi*	58.33±1.82	53.57±0.23	56.25±1.06	5.59±0.93	4.65±0.69	5.19±0.61	3.4±1.22	1.33±0.16	2.54±0.71
Section *Nigri*	66.66±1.36	67.86±0.33	67.19±0.79	7.82±0.96	6.01±0.72	7.02±0.63	3.92±0.91	1.82±0.22	2.99±0.53
Section *Circumdati*	27.77±0.05	39.28±0.09	32.81±0.05	2.30±0.33	5.83±1.75	4.15±0.86	0.73±0.03	0.91±0.06	0.82±0.03
Section *Terri*	11.11±0.13	25.00±0.09	17.18±0.08	9.48±1.57	3.69±0.34	5.79±0.76	1.33±0.09	0.86±0.06	1.03±0.05
Eurotium	30.55±0.89	60.71±0.39	43.75±0.46	7.04±1.82	5.43±1.14	6.07±1.02	2.30±0.59	1.57±0.26	1.86±0.31
Penicillium	66.66±0.28	60.71±0.25	64.06±0.19	4.09±0.49	5.41±0.68	4.64±0.41	1.61±0.19	1.57±0.17	1.59±0.13
Fusarium	97.22±1.67	92.86±1.71	95.31±1.21	24.35±2.81	22.74±2.08	23.66±1.82	9.24±1.11	7.66±1.14	8.57±0.80
Alternaria	83.33±3.36	78.57±0.64	81.25±2.36	23.71±3.53	8.01±1.01	17.31±2.28	10.51±2.24	2.53±0.43	7.32±1.39
Curvularia	72.22±0.93	85.71±1.06	78.12±0.71	9,11±1.45	15.93±1.94	12.38±1.25	3.15±0.62	4.83±0.71	3.96±0.47

Cladosporium	27.77±0.22	39.28±0.19	32.81±0.14	3.94±0.44	2.98±0.31	3.44±0.27	1.20±0.14	1.03±0.13	1.11±0.09
Drechslera	69.44±0.44	89.28±0.72	78.12±0.41	6.86±1.02	9.86±1.16	8.36±0.78	2.58±0.29	3.12±0.48	2.85±0.27
Rhizopus	11.11±0.00	10.71±0.00	10.94±0.00	1.36±0.14	1.98±0.14	1.62±0.11	0.66±0.00	0.66±0.00	0.66±0.00
Other genera	88.89±2.26	100±1.32	93.75±1.37	19.59±2.37	22.74±2.32	21.06±1.66	8.54±1.51	6.93±0.88	7.79±0.91

[1]Frequency (%) =Number of samples of occurrence of a genus*100/ total number of samples.

[2]Relative density (%) = number of isolated of a genus *100/ total number of isolated fungi

[3]Average percent (%) = Number of infected kernels by a mold in each sample * 100/ total number of kernels

Table4. Identification of *Aspergillus* section *Flavi* and section *Nigri* isolates

Species	Tunisia		Egypt		Total	
	N	Incidence (%)	N	Incidence (%)	N	Incidence (%)
Aspergillus flavus	51	91.07	31	88.57	82	90.11
Aspergillus parasiticus	5	8.93	4	11.43	9	9.89
Aspergillus section *Flavi*	56		35		91	
Aspergillus niger	89	38.2	30	26.32	119	34.3
Aspergillus tubingensis	57	24.46	23	20.18	80	23.05
Other *Aspergillus niger* aggregate	87	37.34	61	53.51	148	42.65
Aspergillus section Nigri	233		114		347	

N: Isolates number