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Original article

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

Ines Jedidi [a,*], Carlos Soldevilla [b], Amani Lahouar [a], Patricia Marín [c], María Teresa González-Jaén [c], Salem Said [a]

[a] Laboratory of Biochemistry, Faculty of Medicine of Sousse, University of Sousse, Sousse, Tunisia
[b] UD de Zoología, Enfermedades y Plagas Forestales, ETSI Montes, Forestal y del Medio Natural, Universidad Politécnica de Madrid (UPM), Madrid, Spain
[c] Department of Genetics, Faculty of Biology, Complutense University of Madrid (UCM), Madrid, Spain

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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

* Corresponding author at: University of Sousse, Faculty of Medicine of Sousse, Laboratory of Biochemistry, Mohamed El Karoui Street, 4002 Sousse, Tunisia.
E-mail addresses: jedidi.ines@gmail.com (I. Jedidi), carlos.soldevilla@upm.es (C. Soldevilla), amanilahouar@yahoo.fr (A. Lahouar), patmarin8149@bio.ucm.es (P. Marín), tegonja@ucm.es (M.T. González-Jaén), salem.said@famso.rnu.tn (S. Said).
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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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2.3.2. Species specific PCR detection assays

The suitability of genomic DNA for PCR amplification and the presence of fungal DNA were tested in all the DNA samples by a PCR assay using universal primers 5.8S1/5.8S2 (Gil-Serna et al., 2009). Subsequently, the species specific PCR assays described in Table 1 were performed using the conditions described in previous works indicated therein. In all cases, amplification reactions were carried out in volumes of 25 μL containing 100 ng of template DNA, 1 μL of each primer (20 μM), 2.5 μL of 10× PCR buffer, 1 μL of $MgCl_2$ (50 mM), 0.2 μL of dNTPs (100 mM) and 0.15 μL of Taq DNA polymerase (5 U/μL) supplied by the manufacturer (Biotools, Madrid, Spain). PCR was performed in a thermocycler (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany). PCR products were detected in different concentration agarose ethidium bromide gels, depending to the amplicon size, and in TAE 1× buffer (Tris–acetate 40 mM and EDTA 1.0 mM). A 100 bp DNA ladder (MBI Fermentas, Vilnius, Lithuania) was used as molecular size marker.

2.3.3. Amplification and sequence analysis of diagnostic genomic regions

The informative partial sequence of TEF-1α was amplified for those *Fusarium* strains negative for the PCR assays indicated above, using the protocol and primer set described by O'Donnell et al. (1998). In the case of the *Aspergillus* strains other than *Flavi* and *Nigri* sections, the ITS region (ITS1-5.8S-ITS2) of the fungal genomic rDNA was amplified using universal primers ITS1/ITS4 (White et al., 1990) and the protocol described by Henry et al. (2000). The amplified products obtained were purified using the Ultra-CleanTM PCR Clean-UpTM kit (MoBio Laboratories Inc., Carlsbad, CA, USA), and sequenced, in both directions, using the ABI 3730 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions in the Genomic and Proteomic Unit of the Complutense University of Madrid (Spain). Sequences were edited and aligned by BioEdit Sequence Alignment Edition (Ibis Therapeutics, Carlsbad, CA,USA). These sequences were then compared with sequences available from the databases.

3. Results and discussion

3.1. General internal mycoflora

A total of 3690 fungal strains were isolated, representing the internal mycoflora from 31 and 34 samples collected at harvest and stored facilities, respectively. The results of their identification and distribution are shown in Tables 2–4.

The samples studied might be considered representative of the main areas where wheat, barley and maize are cultivated in Tunisia. The results indicate differences in the contamination patterns depending on the cereal species, particularly in the case of maize (Table 3). Barley samples showed the highest percentages of contamination by *Penicillium*, *Aspergillus*, *Eurotium* and *Alternaria*. The highest values of contaminated wheat samples were for *Alternaria*, *Eurotium* and *Aspergillus*. The highest values in maize were reached by *Aspergillus* followed, at quite lower values, by *Eurotium* and *Penicillium*. Barley and wheat samples showed an array of fungal species more diverse than maize. Genus *Ulocladium* and *Curvularia* were not detected in maize samples. Additionally, maize samples had lower relative amount of the class 'Other genera' than barley and wheat. Environmental conditions of maize cultivation differ from barley or wheat, being the more sensitive period for fungal growth later in the year when temperatures are higher and humidity lower in Mediterranean regions. Additionally, maize has higher humidity than barley or wheat at harvest needing further treatment to achieve safer humidity levels in order to prevent fungal growth.

The results of the relative density of fungal genera in the samples and average percentage of infected grains per sample (Table 3) confirmed the different pattern described above for maize when compared to barley and wheat. Maize samples contained *Aspergillus* at the highest density, followed by *Penicillium* and *Eurotium* at quite lower values. Barley and wheat showed the highest fungal density for *Eurotium*, *Alternaria*, *Penicillium* and *Aspergillus*, respectively. All these fungal genera, occurring at high values, are able to grow at conditions of low humidity and they are typically considered as storage fungi (Logrieco et al., 2003). Similar results, concerning the dominance of *Alternaria* genus in wheat grains, were shown in a Tunisian previous study (Belkacem-Hanfi et al., 2013), However, this finding differed from others obtained in Morocco, where *Aspergillus* was the dominant genus isolated from Moroccan wheat samples (Hajjaji et al., 2006). The presence of different

Table 2
General distribution of fungal strains isolated from cereal samples.

Cereal samples (n°)	Average contaminated seeds*/sample	Total fungal strains	Average n° fungal strains/sample
WH (11)	79.64 ± 2.71	1024	93.09 ± 2.80
BH (10)	67.60 ± 2.84	836	83.60 ± 3.80
MH (10)	60.00 ± 5.37	708	70.80 ± 7.44
WS (13)	25.38 ± 5.01	348	26.77 ± 5.43
BS (10)	33.60 ± 4.49	358	35.80 ± 5.05
MS (11)	30.27 ± 6.98	416	37.82 ± 9.11

W = Wheat; B = Barley; M = Maize; S = Storage; H = Harvest.

* contaminated seeds out 100 analyzed per sample, ± mean standard error (SE = $s/\sqrt{N}$).

Table 1
Species-specific PCR assays.

Primer pairs	Target sequence	Amplicon size	Specificity	References
FLA1	5′-TAGGGTTCCTAGCGAGCC-3′	500 bp	*A. flavus*	González-Salgado et al. (2008)
FLA2	5′-GGAAAAAGATTGATTTGCGTTC-3′			
PAR1	5′-GTCATGGCCGCCGGGGGCGTC-3′	430 bp	*A. parasiticus*	Sardiñas et al. (2010)
PAR2	5′-CCTGGAAAAAATGGTTGTTTTGCG-3′			
ITS1	5′-TCCGTAGGTGAACCTGCGG-3′	420 pb	*A. niger aggregate*	González-Salgado et al. (2005)
NIG	5′-CCGGAGAGAGGGGACGGC-3′			
CAR1	5′-GCATCTCTGCCCCTCGG-3′	420 pb	*A. carbonarius*	Patiño et al. (2005)
CAR2	5′-GGTTGGAGTTGTCGGCAG-3′			
Fgr-F	5′-GTTGATGGGTAAAAGTGTG-3′	500 pb	*F. graminearum*	Jurado et al. (2005)
Fgc-R	5′-CTCTCATATACCCTCCG-3′			
VERT-2	5′-CACCCGCAGCAATCCATCAG-3′	700 bp	*F. verticillioides*	Patiño et al. (2004)
Fps-F	5′-CGCACGTATAGATGGACAAG-3′			
Fp3-F	5′-CGGCCACCAGAGGATGTG-3′	230 bp	*F. proliferatum*	Jurado et al. (2006a)
Fp4-R	5′-CAACACGAATCGCTTCCTGAC-3′			
Feq-F	5′-GGCCTGCCGATGCGTC-3′	990 pb	*F. equiseti*	Jurado et al. (2005)
Feq-R	5′-CGATACTGAAACCGACCTC-3′			

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Table 3
Internal mycoflora of wheat, barley and maize samples from Tunisia.

	Relative frequency[a]			Average relative density[b,*]			Average percentage of infected grains[c,*]		
	Wheat	Barley	Maize	Wheat	Barley	Maize	Wheat	Barley	Maize
Aspergillus	54.17	70.00	76.19	7.10 ± 1.95	10.05 ± 3.11	44.84 ± 8.11	4.08 ± 1.02	5.8 ± 1.76	27.05 ± 6.39
Section *Flavi*	45.83	50.00	66.67	3.13 ± 0.79	7.74 ± 3.09	30.36 ± 6.29	1.67 ± 0.45	4.00 ± 1.65	19.24 ± 4.92
Section *Nigri*	33.33	25.00	47.62	3.98 ± 1.47	2.05 ± 0.92	14.37 ± 4.96	2.42 ± 0.82	1.60 ± 0.75	7.71 ± 3.31
Other *Aspergilli*	0.00	10.00	9.52	0.00 ± 0.00	0.26 ± 0.18	0.21 ± 0.14	0.00 ± 0.00	0.20 ± 0.14	0.19 ± 0.13
Penicillium	41.67	75.00	38.10	9.39 ± 3.50	13.08 ± 3.26	11.74 ± 5.02	6.34 ± 3.42	9.2 ± 2.91	4.48 ± 1.84
Fusarium	9.52	25.00	19.05	0.21 ± 0.14	1.40 ± 0.71	1.68 ± 0.79	1.17 ± 0.12	1.10 ± 0.55	0.67 ± 0.35
Eurotium	62.5	65.00	42.86	24.80 ± 4.51	17.70 ± 5.97	9.05 ± 4.43	17.17 ± 4.22	6.9 ± 2.00	4.67 ± 2.58
Alternaria	70.83	65.00	14.29	9.81 ± 2.40	13.37 ± 3.34	0.85 ± 0.49	5.42 ± 1.30	8.60 ± 2.24	0.29 ± 0.16
Curvularia	20.83	20.00	0.00	1.07 ± 0.59	1.41 ± 0.75	0.00 ± 0.00	0.92 ± 0.59	1.10 ± 0.59	0.00 ± 0.00
Epicoccum	37.5	40.00	14.29	7.17 ± 2.68	6.76 ± 2.72	4.39 ± 3.66	2.00 ± 0.88	3.80 ± 1.57	2.19 ± 1.90
Cladosporium	29.17	25.00	20.00	2.51 ± 1.02	2.63 ± 1.55	1.28 ± 0.66	0.97 ± 0.34	1.80 ± 1.20	0.57 ± 0.28
Ulocladium	8.33	20.00	0.00	0.19 ± 0.12	0.49 ± 0.23	0.00 ± 0.00	0.17 ± 0.12	0.40 ± 0.18	0.00 ± 0.00
Rhizopus	4.17	25.00	14.29	4.13 ± 3.79	8.13 ± 4.51	9.84 ± 5.48	0.83 ± 0.83	3.20 ± 1.45	5.71 ± 3.94
Chaetomium	25.00	25.00	14.29	2.49 ± 0.91	0.72 ± 0.30	0.83 ± 0.48	1.00 ± 0.43	0.60 ± 0.26	0.29 ± 0.16
Other genera	91.67	85.00	76.19	31.12 ± 3.85	24.25 ± 4.29	15.50 ± 5.41	18.17 ± 3.18	17.20 ± 3.34	7.62 ± 2.71

[a] Relative frequency of contaminated samples (%) = Number of samples contaminated with a genus × 100/Total number of samples.
[b] Relative density per sample (%) = Number of isolates of a genus contaminating a sample × 100/Total number of isolates.
[c] Percentage of infected grains per sample (%) = Number of infected grains by a genus in each sample × 100/Total number of grains.
[*] Mean value ± Mean Standard Error (SE = $s/\sqrt{N}$).

Table 4
Distribution of toxigenic mycoflora in harvest and postharvest samples of wheat, barley and maize.

		WS	WH	BS	BH	MS	MH
Relative frequency	*Aspergillus*	46.15	63.64	70.00	70.00	54.55	100.00
	Section *Flavi*	46.15	45.45	70.00	30.00	45.45	90.00
	Section *Nigri*	15.38	54.55	10.00	40.00	27.27	70.00
	Penicillium	38.46	45.45	70.00	80.00	54.55	20.00
	Fusarium	0.00	18.18	0.00	50.00	18.18	20.00
	Alternaria	53.85	90.91	30.00	100.00	18.18	10.00
Average relative density[*]	*Aspergillus*	9.10 ± 3.44	5.11 ± 1.35	14.53 ± 5.71	5.57 ± 1.97	22.37 ± 8.37	67.32 ± 9.64
	Section *Flavi*	4.61 ± 1.29	1.65 ± 0.71	13.70 ± 5.60	1.79 ± 1.04	11.85 ± 4.31	48.87 ± 8.81
	Section *Nigri*	4.50 ± 2.58	3.46 ± 1.17	0.83 ± 0.83	3.27 ± 1.61	10.52 ± 5.60	18.22 ± 8.50
	Penicillium	7.52 ± 2.90	11.26 ± 6.94	9.42 ± 2.60	16.75 ± 5.92	19.60 ± 8.79	3.87 ± 3.47
	Fusarium	0.00 ± 0.00	0.43 ± 0.29	0.00 ± 0.00	2.80 ± 1.30	1.82 ± 1.11	1.54 ± 1.18
	Alternaria	10.65 ± 4.05	8.97 ± 2.38	6.50 ± 4.95	20.24 ± 3.48	1.28 ± 0.86	0.42 ± 0.42
Average percentage of infected grains[*]	*Aspergillus*	3.38 ± 1.56	4.91 ± 1.30	6.80 ± 3.07	4.80 ± 1.87	8.55 ± 3.00	47.40 ± 9.59
	Section *Flavi*	1.69 ± 0.59	1.64 ± 0.70	6.40 ± 3.02	1.60 ± 1.02	5.64 ± 2.54	34.2 ± 7.58
	Section *Nigri*	1.69 ± 1.19	3.27 ± 1.09	0.40 ± 0.40	2.80 ± 1.37	2.91 ± 1.53	13.00 ± 6.51
	Penicillium	1.85 ± 0.95	11.67 ± 7.22	3.80 ± 1.17	14.60 ± 5.28	5.64 ± 2.32	3.20 ± 2.98
	Fusarium	0.00 ± 0.00	0.36 ± 0.24	0.00 ± 0.00	2.20 ± 1.01	0.36 ± 0.24	1.00 ± 0.68
	Alternaria	2.77 ± 1.12	8.55 ± 2.23	0.80 ± 0.44	16.40 ± 2.75	0.36 ± 0.24	0.20 ± 0.20

W = Wheat; B = Barley; M = Maize; S = Storage; H = Harvest.
[*] Mean value ± Mean Standard Error (SE = $s/\sqrt{N}$).

Aspergillus species in freshly harvested cereal grains was previously reported (Riba et al., 2010, 2008). Differences in the local atmospheric variables (temperature and humidity) and between years might cause shifts in the mycoflora composition of cereals reaching harvest as well as conditions during the subsequent storage.

The mycoflora, other than the toxigenic species which it may also include, might play a role in the final array of the toxins produced in the seeds and their relative amount as well as in the deterioration they might produce of the grain quality. Particularly interesting is the case of *Eurotium* which showed a high incidence in this study. The species included in this genus, which have undergone a necessary nomenclatural revision to be now placed within *Aspergillus* genus (Hubka et al., 2013), are considered mostly non toxigenic except for some minor compounds not legally regulated yet (Greco et al., 2015). However, this genus considered highly xerophilic or halophilic, actively contributes to deterioration of stored grain and food products, among other substrates. Additionally, their release metabolic water during substrate colonization creates more favorable environment for other fungal species able to produce mycotoxins. The final outcome regarding composition of fungal population during storage, and hence mycotoxin risk, will further depend on a good quality system of the initial grain conditions and throughout the storage in silos (Belkacem-Hanfi et al., 2013; Magan and Aldred, 2007).

3.2. *Aspergillus* distribution

From the safety point of view, the most relevant fungal species belonged to *Aspergillus* section *Flavi*, followed by Section *Nigri*. *Aspergillus* section *Flavi* showed high percentages of contaminated samples, 66.67%, 50% and 45% in maize, barley and wheat, respectively (Table 3). Relative density was also notably higher in maize than in barley or wheat (Table 3), indicating that maize might represent a considerable higher risk for the highly toxic Aflatoxins (AFs). All the 84 *Aspergillus* section *Flavi* strains analyzed were positive to the *A. flavus* specific assay whereas none showed amplification with the *A. parasiticus* specific assay. Previous studies also indicated the prevalence of *A. flavus* among *Aspergillus* section *Flavi* in cereals and other commodities (Jedidi et al., 2017; Melki-Ben Fredj et al., 2007; Riba et al., 2008). The high contamination of analyzed cereals

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by *A. flavus* strains, able to produce simultaneously AFB1, AFB2, cyclopiazonic acid, sterigmatocystin, among others, suggest the possibility of the co-occurrence of these toxins in Tunisian cereals, but not the AFG1 and AFG2 aflatoxins which are produced by *A. parasiticus* strains (Rodrigues et al., 2012; Wilson et al., 2002).

Concerning *Aspergillus* section *Nigri* (*A. niger* aggregate or *A. carbonarius*), the percentage of maize contaminated samples was higher than wheat or barley; similarly, the relative density of fungal genera and the average percentage of infected seeds per sample were higher in maize (Table 3). Interesting, the values of harvest samples were consistent, and notably higher than those of stored samples (Table 4). As it was addressed above, evolution of the mycoflora during storage depends on the mycoflora composition at harvest, but also on the storage conditions. The PCR assay using primers ITS1 and NIG, which amplify a single fragment of about 420 bp, allowed discrimination of the two species of the *A. niger* aggregate (*A. niger* and *A. tubingensis*) from the rest of the species included in the *Nigri* section of *Aspergillus*. Results showed that all DNA samples, extracted from the 43 *Aspergillus* section *Nigri* strains, amplified with the ITS1/NIG primers, being so *A. niger* aggregate strains. In a previous work performed by Jedidi et al., A. niger aggregate is also the unique species, belonging to *Aspergillus* section *Nigri*, found contaminating Tunisian wheat, barley and maize (Jedidi et al., 2017). Regarding this Section the potential toxin risk is mainly associated to ochratoxin A (OTA), being notably higher in the case of *A. carbonarius* (Medina et al., 2005). However, no strains of *A. carbonarius* were found in any of our samples. In agreement with our results, *A. carbonarius* had not been previously reported as contaminant of Moroccan wheat (Hajjaji et al., 2006). However, in other previously published work, *A. carbonarius* was found contaminating Algerian wheat, but at much lower frequency, comparing to *A. niger* (Riba et al., 2008). Therefore, this is a species to be included in the routine tests searching for potential mycotoxin producing species in cereals.

Sequencing of the ITS1/ITS4 amplification products has permitted to identify the three *Aspergillus* isolates which were not identified as belonging to *Flavi* or to *Nigri* section, using morphological characters. Among these three isolates, one was an *A. sydowii* strain, being isolated from a harvest maize sample (Access Number: MG459153), and two were *A. fumigatus* and *A. nidulans*, isolated from two different harvest barley samples (Access Numbers: MG459154 and MG459155, respectively). Occurrence of *A. nidulans* species has been reported at low frequency in previous study analyzing Moroccan wheat (Hajjaji et al., 2006). *A. fumigatus* was also previously isolated at low incidence in Algerian stored durum wheat (Riba et al., 2008). To our knowledge, *A. sydowii* was not detected in previous known cereal analyzing works but looking to the literature, *A. sydowii* was defined as one of the fungal strains which produce sterigmatocystin when contaminating grains, coffee beans, maize, fruits and grape juice (Amalaradjou and Venkitanarayanan, 2008).

None *Aspergillus* section *Circumdati* isolate was detected in any of analyzed samples. Unlike our results, *A. ochraceus* belonging to section *Circumdati*, was isolated from Moroccan and Algerian wheat (Hajjaji et al., 2006; Riba et al., 2008).

3.3. Fusarium distribution

The second relevant toxigenic genus studied in more detail in this work was *Fusarium*. In contrast with *Aspergillus*, *Fusarium* occurred at much lower relative frequency in samples, 18.18% in wheat and 50% in barley (Table 4), both cases only on samples collected at harvest time. This might suggest that the conventional methods used for fungal isolation, when performed at late stages (harvest time and, particularly in the case of stored seeds) might strongly under represent those species having their most favorable conditions (higher humidity) at earlier times. This is the case of most *Fusarium* species whose favorable conditions are concomitant with wheat/barley post anthesis. The main *Fusarium* species isolated from wheat and barley in this work was *F. equiseti* (Table 5), whose ecophysiological profile might be considered between *F. graminearum* and *F. verticillioides* (Marín et al., 2015), being *F. verticillioides* more tolerant to low water potential and higher temperatures (Marín et al., 2010). This profile is compatible with conditions during maize growth in field even approaching harvest time. This seemed consistent with the detection of *Fusarium* in both stored and harvest maize samples (Table 4) and the identification of *Fusarium* isolated from maize as *F. verticillioides* (Table 5). We suggest that direct species specific PCR assays on stored samples or on samples collected at late stages of cereal growth might provide a more sensitive method for detection of early colonizers like *Fusarium* (Gil-Serna et al., 2013).

As previously mentioned, the identification of *Fusarium* species revealed that *F. verticillioides* was the unique species detected in maize, whereas *F. equiseti* (nine out 12 isolates) was prevalent in barley and wheat (Table 5). Additionally, the sequencing of the partial sequence of TEF-1α gene has permitted to identify one *F. oxysporum* (Access Number: MG452940) and two *F. nygamai* (Access Numbers: MG452941 and MG452942) in barley. *F. verticillioides* and *F. nygamai* are fumonisins producers, being the first mostly present in maize where is highly pathogenic and the latter in sorghum (Leslie et al., 2005; Waalwijk et al., 2008). In the case of *F. oxysporum* is generally considered non toxigenic. On the other hand, *F. equiseti* has been commonly detected in barley and wheat cultivated in Mediterranean countries (Balmas et al., 2010; Castellá and Cabañes, 2014; Jurado et al., 2006b; Marín et al., 2012) and seems to belong to a phylogenetically distinct population (Marín et al., 2012) within of the *Fusarium incarnatum-Fusarium equiseti* species complex (FIESC) described by O'Donnell et al. (2009). The mycotoxin profile of this population differs from that previously described for *F. equiseti* strains isolated from Northern Europe (Kosiak et al., 2005; Marín et al., 2012). The group of Spanish *F. equiseti* analyzed produced deoxynivalenol, nivalenol and other trichothecenes at variable levels while no type A trichothecenes could be detected.

A recent study carried out on stored samples of sorghum in Tunisia reported the occurrence of the similar fungal genera described in this work. Interestingly, relative amounts for most of the fungal genera were notably higher than those found in our work, in particular in the case of *Fusarium*, which showed a percentage of contaminated samples above 90% and relative density above 20%, being the most prevalent genus of the mycoflora analyzed in their case (Lahouar et al., 2015). This might suggest that sorghum might be more prone to fungal contamination. Additionally, environmental conditions in field and during storage might have contributed to the final higher levels observed in that

Table 5
Identification of *Fusarium* isolates by PCR assays and/or TEF1α partial sequence, host and location.

No. Strains	Cereal	Origin	[1]PCR assays/[2]*TEF1α*
1	Wheat	Sousse (East)	[1]*F. equiseti*
1	Wheat	Kairouan (Center)	[1]*F. equiseti*
2	Barley	Sousse (East)	[1]*F. equiseti*
1	Barley	Sousse (East)	[2]*F. oxysporum*
2	Barley	Sousse (East)	[2]*F. nygamai*
2	Barley	Kairouan (Center)	[1]*F. equiseti*
1	Barley	Sidi Bouzid (Center)	[1]*F. equiseti*
2	Barley	Beja (North)	[1]*F. equiseti*
4	Maize	Kairouan (Center)	[1]*F. verticillioides*
1*	Maize	Monastir (East)	[1]*F. verticillioides*
1*	Maize	Sousse (East)	[1]*F. verticillioides*

TEF1α: Translation Elongation Factor 1 alpha gene.
* *Fusarium* strains isolated from postharvest samples.

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study. On the other hand, *Fusarium* species detected in both sorghum and wheat/barley samples were quite similar. Moreover, no *F. graminearum* nor *F. culmorum* were detected. This latter species was previously indicated in Tunisian wheat as being the highest pathogenic species causing the *Fusarium* Head Blight (FHB) of durum wheat in sub-humid and higher semi-arid region of Northern Tunisia in 2004. Several other *Fusarium* species causing the disease were also detected with significant differences in aggressiveness depending on the species considered, among which we cite *F. avenaceum, F. equiseti, F. pseudograminearum, F. acuminatum and F. sporotrichoides* (Fakhfakh et al., 2011; Gargouri-Kammoun et al., 2009). Differences in the climatic and the environmental conditions in field among locations and years might be responsible for differences in *Fusarium* diversity and incidence in the studies compared. In particular, some reports point out that *F. verticillioides, F. proliferatum* and *F. equiseti* might tolerate better low water potential conditions than *F. graminearum* (Marín et al., 2015, 2012). Therefore, it would be useful to determine the occurrence and prevalence of certain *Fusarium* species at these early critical stages in order to predict which toxin or toxins could be expected and to decide if control treatments should be applied. Additionally, species specific toxigenic fungal detection could also be useful at harvest time and/or in subsequent postharvest stages.

4. Conclusions

To the best of our knowledge, the present work reported the first report about the most common fungal genera contaminating wheat, barley and maize consumed in Tunisia, and this was the first attempt to detect unambiguously the different species of both *Aspergillus* and *Fusarium* groups using molecular methods. It can be concluded that cereal grains consumed in Tunisia were contaminated by several fungal genera, and *Aspergillus* species were the most common group. The isolated *Aspergillus* strains were all identified by molecular methods as *A. flavus* and *A. niger* aggregate. This suggested the possibility of the occurrence of their mycotoxins (basically AFs and, to a lesser extent OTA) in Tunisian cereals. Results of this survey should raise our awareness to provide more data about the fungal diversity, infecting cereals with further works with a higher number of samples giving a special attention to mycotoxigenic species by determining the appropriate fungicides to use in the fields and the appropriate environmental conditions to apply in the storage location, in order to avoid their proliferation and their mycotoxin entering the food chain. The DNA based assays used in this work, namely species specific PCR assays and sequence of target diagnostic genomic regions, represent a useful strategy in terms of time of analysis, sensitivity and specificity in comparison with the conventional methods of fungal identification.

Authors' contribution

Ines Jedidi contributed to conception of the study, collected the samples, performed the practical and statistical analysis and wrote the manuscript. Carlos Soldevilla and Amani Lahouar contributed in the analysis of the mycoflora. Patricia Marín contributed in the molecular identification of the *Aspergillus* and *Fusarium* species. María Teresa González-Jaén and Salem Said contributed to conception of the study and corrected the manuscript.

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Conflict of interest

All the authors of this study declare the absence of any potential conflicts of interest.

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