



Isolation and molecular identification of mycotoxin producing fungi in durum wheat from Morocco

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Abstract

In the present study, a total of 1053 isolates of fungi were isolated from eighty one (81) samples of durum wheat collected in seven areas in Morocco (Khemiset; Béni-Mellal; Settat; Fès-Meknès; Skhirat-Témara; Marrakech; and Rabat-Salé). The molecular identification of isolates was performed by a PCR-based method. Selected fungi strains were then studied for their potential to produce mycotoxins especially ochratoxin A (OTA) and deoxynivalenol (DON). The results of biodiversity showed that the most abundant species were found belonging to *Alternaria* spp. (86.2%), *Fusarium* spp. (71.1%), and *Aspergillus* spp. (14%). Results of LC analysis indicated also that 27 out of 980 isolates (2.75 %) of *Fusarium* spp. produced the toxin DON. The maximum level of DON found (5.22 µg/kg) was produced on a wheat sample from "Settat" area. These results are in agreement with previous data where we reported the presence of DON by LC on the ground wheat (1.013 µg/kg). Results of LC analysis, showed also that OTA was produced by 8 of 43 total isolates (18.6%) of *Aspergillus* section *Nigri*. The maximum level of OTA produced found also in a wheat sample from Settat area (0.72 µg/kg). Molecular identification of the isolates showed the presence of *F. graminearum* (11.1%), *F. equiseti* (61.1%), *F. ecuminatum* (5.5%), *F. sporotrichioides* (5.5%), *F. verticillioides* (11.1%), *F. coeruleum* (5.5%) and *F. oxysporum* (5.5%). The identification of *Aspergillus* species showed the abundance of *A. flavus* (41.1%), *A. niger* (35.6%), *A. tubingensis* (15.1%) and *A. niger aggregate* (8.2%).

1. Introduction

Cereals are susceptible to fungal attack both in the field and during storage. It may cause economic losses at all levels of food and feed production chain [1]. The fungal species that mainly contaminate the crops belong to genera *Fusarium*, *Aspergillus*, *Alternaria*, *Penicillium* and *Claviceps* [2]. These fungi may produce secondary metabolites, known as mycotoxins. Wide year to year fluctuations in the levels of mycotoxins in cereals are observed, the main factors favoring fungal invasion and growth being humidity and temperature. Most of *Fusarium* species can cause a Fusarium head blight (FHB) disease by a production of a plethora of mycotoxins [3]. According to [4, 5 and 6], the main causal agents of FHB in Europe are *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae* and *Microdochium nivale*.

Many mycotoxins were initially discovered after they have had caused a variety of subacute health problems in animals and humans as a result of the consumption of contaminated products. Mycotoxins produced may cause human and animal toxicoses, the principal symptoms are nausea, lethargy, vomiting, digestive and haemolytic disorders, impairment of both humoral and cellular immune responses, and nervous disorders [5]. With the development and application of good storage and processing practices, the mycotoxin contamination and occurrence is considerably reduced, the actual concern being more focused on chronic effects at low levels of exposure. In this regard, several mycotoxins have been classified by the International Agency for Research on Cancer (IARC) as confirmed or potential (probable and possible) human carcinogens [7].

The trichothecene type B deoxynivalenol (DON) or vomitoxin is a mycotoxin produced by several *Fusarium* species, mainly *F. culmorum* and *F. graminearum*, which are abundant in cereal crops [8, 9 and 10].

This mycotoxin is the most prevailing *Fusarium* mycotoxin worldwide [11]. To ensure food and feed safety, the European Commission has set maximum threshold values for some mycotoxins in unprocessed cereals for human consumption [12] and guideline limits for *Fusarium* mycotoxins in animal feed. The thresholds for (DON) content are 0.20 mg/kg for processed cereal-based food for infants and baby food; 0.50 mg/kg for bread, biscuits, cereal snacks and breakfast cereals and 0.75 mg/kg for cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for human consumption. For unprocessed cereals, the threshold is 1.25 mg/kg.

Ochratoxin A (OTA) is part of a group of mycotoxins produced as secondary metabolites by several fungi of the *Aspergillus* or *Penicillium* genus. The chemical structure of OTA represent a pentaketide composed of dihydroisocoumarine linked to β -phenylalanine. OTA has been shown to be nephrotoxic, hepatotoxic, teratogenic and immunotoxic to several species of animals and is known to cause kidney and liver tumors in mice and rats [13]. This toxin is considered to be involved in severe kidney pathologies, possibly linked to urinary tracts tumors [14], and has been classified by the (IARC) as a possible carcinogen to humans (group 2B) [7]. According to the 2002 report on the assessment of dietary intake of OTA by the population of the EU member states [15], this mycotoxin has been detected in a variety of food commodities such as cereals, oleaginous seeds, coffee beans, wine, meat, cocoa and spices.

North Africa, where wheat consumption in the form of couscous, pasta, macaroni, spaghetti, bread, and frik is a cultural tradition, the safety assessment is very important. The mycobiota of wheat and wheat products was found to be dominated by *Aspergillus* sections *Nigri* and *Flavi* species [16, 17 and 18].

In continuity with previous study on hard durum wheat, which found that durum wheat produced and consumed in Morocco is little contaminated with DON. [19], this work is aiming to identify fungi isolated from durum wheat in different area from Morocco using molecular biology tests (PCR) and to evaluate the deoxynivalenol (DON) and ochratoxin A (OTA) production capacity of isolates of *Fusarium* and *Aspergillus* species.

2. Materials and methods

2.1 Sampling

Eighty one (81) samples of durum wheat were collected from different markets or directly from farms in seven areas of Morocco. The weight of each sample was 200 g. Some of the samples were taken from the “Office National Interprofessionnel des Céréales et des Légumineuses” (ONICL) and The “Office National de Sécurité Sanitaire des Produits Alimentaires” (ONSSA). The distribution of the collected samples is shown in table 2. The samples were packed in plastic bags, then stored at 4°C prior to analysis.

2.2 Chemicals and Reagents

Mycotoxin standards, including DON and OTA were supplied by Sigma (Sigma–Aldrich, Alcobendas, Spain). Acetonitrile, methanol and ethanol were purchased from J.T. Baker (Deventer, The Netherlands). Benzene and n-hexane were purchased from Merck (Damstadt, Germany). All solvents were LC grade. Filter papers (Whatman No. 1) and glass microfiber filters (Whatman GF/ A) were purchased from Whatman (Maidstone, UK). Immunoaffinity columns (IAC), DON (DONPREP) and extracts clean-up were purchased from R-Biopharm (Rhone LTD Glasgow, UK). Pure water was obtained from a milli-Q apparatus (Millipore, Billerica, MA, USA) and was used when water was required. Phosphate buffer saline (PBS) was prepared with potassium chloride (0.2 g) (Panreac, Castellar del Valles, Spain), potassium dihydrogen phosphate (0.2 g) (Sigma), disodium phosphate anhydrous (1.16 g) (Panreac) and sodium chloride (8.0 g) (J.T Baker) in 1 L of pure water; the pH was brought to 7.4.

2.3 Mycobiota determination

The study of the mycobiota present in the samples was carried out by determination of internal fungi. For isolation of the internal mycobiota a subsample of 200 kernels of each sample was surface disinfested in a 3% aqueous solution of sodium hypochlorite for 2 minutes, and rinsed twice with sterile distilled water. The kernels were aseptically plated in Potato dextrose agar (PDA) (5 kernels/plate). Moreover, malachite green agar (MGA 2.5) (11.25 g of peptone, 0.75 g of KH_2PO_4 , 0.375 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 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 [20-22]. Plates were incubated at 25°C for 7 days (PDA) and 10 days (MGA 2.5). After incubation, they were examined for fungal growth, and the moulds present on the kernels were identified to genus level using the methods of Pitt and Hocking (1997). *Aspergillus* spp. and *Penicillium* spp. isolates were transferred to PDA, Czapek (Cz) and Czapek yeast extract agar (CYA) plates. *Fusarium* sp. isolates were transferred to Carnation

leaf agar (CLA). The isolation frequency and the relative density of genera and species were calculated according to [23] as follows:

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

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

$$\text{average (\%)} = \frac{\text{Number of infected grains of a genus or species}}{\text{Total number of grains}} \times 100$$

After incubation, *Fusarium* strains were isolated on the (CYA) to study the capacity of DON production and were grouped according to morphological and cultural characteristics. The groups were also identified using the method of the medium (CLA).

The *Aspergillus* strains were isolated from all the media PDA, Cz and CYA for studying their capacity to produce ochratoxin A.

2.4 Molecular identification of *Fusarium* and *Aspergillus* isolates.

The identification of mycotoxigenic *Fusarium* and *Aspergillus* isolates present in the samples of wheat was done by molecular biology methods.

2.4.1 DNA extraction

Cultures were grown for 2 days at 27°C on 500 µl of Czapeck's yeast medium. Mycelium was recovered after 10 min of centrifugation at 17500 xg and 300 µl of extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added. The mycelium suspension was vortexed with five 2.8 mm stainless steel beads (Precellys, Bertin Technologies) during 10 minutes. After centrifugation at 17500 xg for 10 min, 150 µl of 3 M sodium acetate (pH 5.2) were added to the supernatant. The supernatant was incubated at -20°C for 10 more minutes and centrifuged (17500 xg, 10 min). The DNA-containing supernatant was transferred to a new tube and nucleic acids were precipitated by adding 1 volume of isopropyl alcohol. After 5 minutes of incubation at room temperature the DNA suspension was centrifuged (17500 xg, 10 min).

The (DNA) pellet was washed with 70% ethanol to remove residual salts. Finally, the pellet was air-dried and the DNA was resuspended in 50 µl of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA).

2.4.2 DNA Amplification and Sequencing

Specific PCR assays were carried out using primers FLAVIQ1 (5' GTCGTCCTCTCCGG 3') and FLAQ2 (5' CTGGAAAAGATTGATTTGCG 3') for *A. flavus* [24], PAR1 (5' GTCATGGCCGCCGGGGGCGTC 3') and PAR2 (5' CCTGGAAAATGGTTGTTTTGCG 3') for *A. parasiticus* [25], [26], AcKS10R (5'-CCCTGATCCTCGTATGATAGCG-3') and AcKS10L (5'-CCGGCCTTAGATTTCTCTCACC-3') for *A. carbonarius* [27], NIG1 (5'-GATTCGACAGCATTT(CT/TC)CAGAA-3') and NIG2 (5'-AAAGTCAATCACAATCCAGCCC-3') for *A. niger* and TUB1 (5'-TCGACAGCTATTTCCCCCTT-3') and TUB2 5'-TAGCATGTATATCACGGGCAT-3') for *A. tubingensis* [28].

The primer pairs BT2A/BT2B [29] and EF-1/EF-2 [30] were also used to obtain the sequence of beta-tubulin and elongation factor sequences, respectively, in order to identify *Fusarium* isolates. PCR reactions were performed in 50 µl as the final volume, containing 50 ng of DNA, 50 mM KCl, 10 mM Tris-HCl, 80 µM (each) dNTP, 1 µM of each primer, 2 mM MgCl₂ and 0.5 U of DNA polymerase (DFS-Taq DNA polymerase, BIORON, Germany). The reaction mixtures were incubated in a thermocycler (Applied Biosystem GeneAmp 2700) for 35 cycles consisting of 30 s at 95°C, 45 s at 60°C and 1 min at 72°C. PCR products were cleaned with the UltraClean PCR Clean-up DNA Purification kit (MoBio, USA). The PCR purified products were directly sequenced by the company Macrogen (Seoul, Korea). Then Blast n-searches in GenBank permitted the identification of the level of identity with known sequences, and subsequently the species corresponding to the isolates.

2.5 DON and OTA production ability

2.5.1 OTA-producing ability

The isolates belonging to *Aspergillus* and *Penicillium* genera were evaluated for OTA-producing ability using a previously described high performance liquid chromatography (HPLC) screening method [31]. Briefly, the 73

isolates were grown in plates containing (CYA) [32] and incubated at 25°C for 7 days. From each culture, three agar plugs were removed from different points of the colony and extracted with 1 mL of methanol. The obtained extracts were filtered and analysed 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 0.21 µg/kg.

2.5.2 DON-producing ability

The isolates belonging to *Fusarium* genus were evaluated for DON-producing ability. So, 980 *Fusarium* isolates were inoculated on (CYA) and incubated at 25°C for 7 days.

DON was extracted by a variation of a simple method [31]. Briefly, three agar plugs (diameter: 6 mm) were obtained from the inner, middle and outer area of each colony of potential DON-producers grown on (CYA) plates, and were introduced in a vial containing 1 ml of the mobile phase (94% H₂O, 3 % acetonitrile, 3% methanol). After the agitation and a rest of 60 min at room temperature, the extract was analyzed by HPLC after stirring and filtration with a hydrophilic filter (0.45 µm).

The toxin (DON) was detected and quantified by using an LC system (Waters 2695, separations module, Waters, Milford, USA) and a column (Waters Spherisorb 5µ m 4.6 x 150 mm analytical column). The mobile phase (water/acetonitrile/methanol; 94/3/3, v/v/v) was pumped at 1.2 mL/min. The temperature of the column oven was 35 °C. The absorbance detector 2487 (Waters, Milford, USA) was set at 220 nm. The retention time of (DON) under these conditions was 16 min. The detection limit was 62.5 µg/kg. Quantification was always achieved with a software integrator (Empower, Milford, MA, USA). The (DON) was quantified on the basis of the (HPLC) absorbance response compared with that of a range of analytical standards.

2.6 Water activity (aw)

AquaLab serie 3 (Decagon devices Inc., WA, USA) was used to determine aw in wheat sample. The patented chilled-mirror dew-point sensor measures water activity between 0.030-1.000aw with a ±0.003aw accuracy in less than five minutes. aw was measured for all samples of seven areas.

2.7 Chromatographic conditions

2.7.1 Deoxynivalenol (DON)

The toxin was detected and quantified by using an LC system (Waters 2695, separations module, Waters, Milford, USA) and a column (Waters Spherisorb 5µm 4.6 x 150mm analytical column). The mobile phase (water/acetonitrile/methanol; 94/3/3, v/v/v) was pumped at 1.2 mL/min. The temperature of the column oven was 35°C. The absorbance detector 2487 (Waters, Milford, USA) was set at 220 nm. The retention time of (DON) under these conditions was 16 min. The detection limit was 62.5 µg/kg. Quantification was always achieved with a software integrator (Empower, Milford, MA, USA). The toxin DON was quantified on the basis of the HPLC absorbance response compared with that of a range of analytical standards.

2.7.2 Ochratoxin A

The equipment used for the (HPLC) detection of (OTA) was a separation Module Alliance 2695 Waters, an analytical column Water Spherisorb 5 mm ODS2, 4.6 _ 250 mm and a Multi k Fluorescence Detector Waters 2475. Excitation and emission wavelengths were set, respectively, at 365 and 455 nm for AFs (0 to 16 min), and 333 and 463 nm for OTA (16 to 25 min). Derivatization of aflatoxins was obtained using a post-column photochemical derivatization device (UVE™ Derivatizer LC Tech, Germany). Mobile phase consisted of methanol, acetonitrile and acetic acid 0.1%, using the following proportions: 27% of methanol, 14% of acetonitrile and 59% of acetic acid 0.1%, until minute 16, and then 50% of methanol and 50% of acetonitrile until the end of the run. The mobile phase flow rate was 0.8 mL/min. The injection volume was 100 µL.

2.8 Validation of analytical method

The analytical methods used were assessed for selectivity, linearity, and precision. Selectivity was checked by injecting 100 µL of mycotoxin standard solution three times before injecting extracted samples and comparing the peak retention times and the fluorescence spectra of the substances that produced these peaks. Standard curves were generated by linear regression of peak areas against concentrations. Precision was established by determining (OTA, DON), in ground wheat and positive strains samples at least by triplicate, in those samples (blank samples) fortified in order to calculate the recovery rates. The limit of detection (LOD) was considered to be three fold the signal of blank noise, and the limit of quantification (LOQ) was calculated as 3x LOD. Method performance characteristics for DON, OTA are summarized in Table 1. These values are in accordance to performance criteria established by Commission Regulation (EC) No. 401/2006 [12].

Table 1: Method performance for detection of deoxynivalenol (DON) and ochratoxin A (OTA) in media culture.

	LODa (ug/kg)	LOQb (ug/kg)	n	Spiking level (ug/kg)	Recovery (%) ^c	RSDrd (%)
OTA	0.2	0.6	5	2.5	74 ± 16	21
			5	5.0	103 ± 13	95
			5	7.5	± 12	13
DON	100	300	5	100	124 ± 8	6
			5	500	86 ± 10	12
			5	1000	99 ± 3	3

a LOD = Limit of detection.

b LOQ = Limit of quantification.

c Mean value ± standard deviation.

d RSDr = relative standard deviation.

3. Results and discussion

3.1 Data treatment Biodiversity

Fungal results associated with durum wheat grain collected from Moroccan markets and farms are presented in Table 2. Macroscopic and microscopic examinations of the isolates led us to identify the following genera listed in decreasing order of frequency *Alternaria*. 86.2%, *Fusarium*. 71.1%, *Rhizopus*. 8.06%, *Epicoccum*., 6, 97%, *Penicillium*. 6.38%, *Cladosporium* .4.68%, *Aspergillus* section *Nigri* 4.04%, *Ulocladium*. 2.22%, *Aspergillus* section *Flavi* 2%, *Drechslera*. 1.77%, *Mucor* ., 1.28%, *Curvularia*. 0.45% and other genera 7.32%.

According to the results obtained in our study regarding mycotoxin producing genera, *Fusarium*. was present in all samples collected from the seven areas, the most infected areas being Skhirat-Témara, Khmisset and Béni-mellal.. The values were 86 %, 84 % and 76.25 %, respectively. The second genus of interest in terms of percentage of contaminated samples was *Aspergillus*, particularly *Aspergillus* section *Nigri* and *Aspergillus* section *Flavi*. Its presence was detected in all samples, although the infection level was not very high. The most contaminated areas by *Aspergillus* section *Nigri* were Skhirat-Témara, Fès-Meknès and Rabat-Salé . The values were 10.5 %, 8.85 % and 2.69 %, respectively (Tables 2). Concerning *Aspergillus flavus* we found a strong presence in the grain from Fès-Meknès (6.92 %) and Skhirat-Témara (3.5 %).

We have noticed a difference in the presence of *Fusarium* and *Aspergillus* in the seven areas of the Kingdom. This difference may be due to the climate conditions (temperature, precipitation, etc.) which changes from one region to another and several factors related to storage parameters such as temperature and humidity. Cereals are highly nutritive substrates rich in starch and protein, promoting fungal invasion, by providing necessary carbohydrates and nitrogen for micromycetes development [33]. According to the obtained results on wheat fungal biodiversity, it can be seen that both field and storage molds are present. The same conclusion resulted from the work of [34]. The matching result is due to the similarity of climate conditions between Morocco and Tunisia. Other similar studies on this topic have found the same fungal genera predominance pattern [35-38].

3.2 Water activity

Among the parameters that help a mold to proliferate and produce mycotoxins is the water activity. Below a certain availability of water (aw) they cannot grow or produce mycotoxins, although some spores survive and may be activated when these conditions are favourable. The optimal water activity values for several fungal species are : aw > 0.95-0.90: *Aspergillus fumigatus*, *Trichoderma* sp., *Stachybotrys chartarum*, *Phialophora* sp., *Alternaria* sp., and *Fusarium* sp, *Phoma* sp. aw from 0.90 to 0.85: *Aspergillus versicolor*, *A. sydowii*, and *A. nidulans*.

aw < 0.85: *A. versicolor*, *A. glaucus*, *Penicillium chrysogenum*, *P. aurantiogriseum*) [35]. According to the found results Table 5, the water activity values are as follows: Rabat-Salé (0.589 ± 0.054), Marrakech (0.566 ± 0.064), Skhirat-Témara (0.538 ± 0.067), Fès-Meknès (0.511 ± 0.034, Settat (0.556 ± 0.047), Khmisset (0.635 ± 0.053), Béni-mellal (0.592 ± 0.0168). These values are within the range proposed to prevent fungal growth during wheat storage and commercialization [36]. However, before wheat reaches these conditions of water activity allowing free storage of mycotoxin-free and fungal growth, these mycotoxins have been produced both in the field or while the grain dried. For all of this, it is necessary to know the mycobiota present in the wheat grains with capacity to produce deoxynivalenol and ochratoxin A.

Table 2: Internal mycobiota of wheat samples from different areas of Morocco.

	Rabat- Salé			Marrakech			Skhirat-Témara			Fès-Meknès			Settat			Khmiset			Béni-m	
	(n= 13) a			(n= 12)			(n= 10)			(n= 13)			(n= 11)			(n= 12)			(n= 1	
	a	b	C	a	b	c	a	b	c	a	B	c	a	b	c	a	b	c	a	b
<i>Fusarium</i>	64.62	11.98	4.01	52.08	9.66	3.23	86.00	19.32	6.47	61.92	13.27	4.44	72.73	11.95	4.00	76.25	19.25	6.44	84.00	14.50
<i>Alternaria</i>	77.31	12.8	8.43	79.17	15.52	10.22	78.5	8,92	5.88	82.31	13.64	8.99	95.91	16.77	11.05	90	19.04	12.54	100	13.3
<i>Epicoccum</i>	1.92	6.61	0.1	5.42	12.4	0.19	2	4.13	0.06	2.69	7.44	0.11	3.18	5.79	0.09	19.58	41.32	0.62	14	22.3
<i>Cladosporium</i>	5	15.91	0.17	2.92	13.64	0.15	4.5	13.64	0.15	12.31	42.05	0.46	1.82	4.55	0.05	3.75	3.41	0.04	2.5	6.82
<i>Curvularia</i>	0.38	28.57	0.02	0	0	0	0	0	0	0	0	0	0.91	28.57	0.02	0.83	28.57	0.02	1	14.29
<i>Drechslera</i>	0	0	0	2.08	19.35	0.07	4	29.03	0.11	2.69	25.81	0.1	2.27	16.13	0.06	0.83	6.45	0.02	0.5	3.23
<i>Rhizopus</i>	8.46	17.56	0.28	21.67	38.93	0.63	3.5	5.34	0.09	4.23	8.4	0.14	4.55	7.63	0.12	2.5	4.58	0.07	11.5	17.50
<i>Ulocladium</i>	1.92	19.23	0.06	4.17	19.23	0.06	1.5	0	0	1.92	19.23	0.06	0.91	7.69	0.02	2.08	15.38	0.05	3	19.23
<i>Aspergillus section Flavi</i>	1.15	10	0.05	0	0	0	3.5	20	0.1	6.92	55	0.27	0.91	7.5	0.04	0	5	0.02	1.5	2.5
<i>Aspergillus section Nigri</i>	2.69	11.48	0.09	0.42	1.64	0.01	10.5	37.7	0.28	8.85	36.07	0.27	1.82	4.92	0.04	2.5	4.92	0.04	1.5	3.28
<i>Penicillium</i>	12.69	25.5	0.63	1.67	4.5	0.11	11	33	0.81	15.77	34	0.84	2.73	1	0.02	0.83	2	0.05	0	0
<i>Mucor</i>	0.77	80	0.05	0	0	0	0	0	0	0	0	0	0.45	20	0.01	6.25	0	0	1.5	0
Other genera	11.15	27.33	0.51	4.58	8.67	0.16	14.50	25.33	0.47	4.62	12.67	0.23	5.45	8.67	0.16	5.42	11.33	0.21	5.50	6.00

a: Frequency (%) = number of samples of occurrence of a genus x 100/total number of samples.

b :Relative density (%) = number of isolated of a genus x 100/total number of isolated fungi.

c : Average percent (%) = Number of infected kernels by a mold in each sample x100/total number of kernels.

Table 3: Molecular identification of *Fusarium* and *Aspergillus* species present in Moroccan wheat.

	Rabat-Salé	Marrakech	Skhirat-Témara	Fès-Meknès	Settat	Khmiset	Béni-mellal
<i>F. graminearum</i>	1	1	-	-	-	-	-
<i>F. culmorum</i>	-	-	-	-	-	-	-
<i>F. equiseti</i>	2	2	-	1	2	-	4
<i>F. langsethiae</i>	-	-	-	-	-	-	-
<i>F. acuminatum</i>	-	-	-	-	-	-	1
<i>F. sporotrichioides</i>	-	-	1	-	-	-	-
<i>F. verticillioides</i>	-	-	-	-	-	-	2
<i>F. coeruleum</i>	-	-	1	-	-	-	-
<i>F. oxysporum</i>	-	-	1	-	-	-	-
<i>F. avenaceum</i>	-	-	-	-	-	-	-
<i>A. tubingensis</i>	1	-	3	6	1	-	-
<i>A. niger</i>	3	-	17	6	-	-	-
<i>A. niger aggregate</i>	-	-	1	5	-	-	-
<i>A. flavus</i>	2	4	7	13	2	1	1

Table 4 : Deoxynivalenol (DON) and Ochratoxin A (OTA) production by isolated.

Areas	Deoxynivalenol (DON)				Ochratoxin A (OTA)			
	Positive strains (frequency %)	Range (µg/Kg)	Mean±RSD** (µg/Kg)	Max level (µg/Kg)	Positive strains (frequency %)	Range (µg/Kg)	Mean±RSD** (µg/Kg)	Max level (µg/Kg)
Rabat- Salé	3 (0.30)	33.3 - 122	74.16±44.75	122	Nd	Nd	Nd	Nd
Marrakech	5 (0.51)	<15.6 - 332	92.268±135.62	332	Nd	Nd	Nd	Nd
Skhirat-Témara	Nd	Nd	Nd	Nd	1 (2.4)	0-0.57	0.285±0.403	0.57
Fès-Meknès	Nd	Nd	Nd	Nd	3 (7.1)	0.081-0.635	0.269±0.316	0.635
Settat	5 (0.51)	45 - 5222	1232.2±2239.11	5222	3 (7.1)	0.123-0.727	0.353±0.326	0.727
Khmiset	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Béni-mellal	5 (0.51)	23.3-332	109.86±126.40	332	Nd	Nd	Nd	Nd

3.3 Mycotoxin-producing ability and molecular identification of *Fusarium* and *Aspergillus* species

A correct identification of the species is needed to assess the risk of contamination of food by mycotoxins. Not all the species of a genus are able to produce mycotoxins and their production level is different... It was noted that the two mycotoxins are present in several cereals. Only 27 from the analyzed strains (980 strains in total) are able to produce DON with a minimum concentration of < 15.6 µg/Kg for the Marrakech area and a maximum concentration level of 5222 µg/Kg in samples of Settat Table 4. The obtained results for the production of DON confirm that of a previous work [41] except for the results of the region of – Fès-Meknès where no producing strains were detected; one sample is contaminated which agrees with the results of an earlier study [19].

Among the identified species, DON producers are mainly *Fusarium graminearum* and *Fusarium coeruleum* [42]. Only two regions were characterized by the presence of these molds (Rabat – Salé and Skhirat-Témara). The *Aspergillus* species isolated and identified from 81 samples from seven areas of Morocco are *A. tubingensis*, *A. niger*, *A.niger aggregate* and *A. flavus*. The most abundant is *Aspergillus flavus* (30 isolates), followed by *A. niger* (26 isolates), *A. tubingensis* (11 isolates) and *A.niger aggregate* (6 isolates) Table 3. Among the identified strains there are ochratoxin and / or aflatoxin producing ones. The ochratoxin A is mainly produced by the genera *Aspergillus* and *Penicillium*, particularly *Aspergillus ochraceus*, *A. carbonarius*, *A. niger* and *Penicillium verrucosum* predominantly found in wheat. Wheat products are the major group of food commodities where this toxin has the greatest impact, due to its usage as staple food for majority of world population [43]. We notice a ochratoxin's production activity among strains collected from samples culture of Skhirat-Témara, Fès-Meknès and Settat which maximum values are 0.57 µg/Kg, 0.635 µg/Kg and 0.727 µg/Kg, respectively. OTA was produced by 4 out of 11 *A. tubingensis* (36.36%) and 3 out of 26 *A. niger* isolates (11.5%). The reported percentages of ochratoxigenic isolates of *A. niger* are quite variable depending on the number of isolates studied and geographical origin [44, 45]. *A. tubingensis* was detected for the first time as a producer of OTA in grape in the work of [46]. The low level of production of ochratoxin by the isolated strain is due to the storage conditions more or less respected compared to the results obtained by other researchers [16, 36 and 45]. *A. tubingensis* produces low amount of OTA [47].

Table 5: Water activity of Moroccan wheat grains.

Moroccan Areas	aw (mean ± SD)
Rabat-Salé	0.589±0.054
Marrakech	0.566±0.064
Skhirat-Témara	0.538±0.067
Fès-Meknès	0.511±0.034
Settat	0.556±0.047

Conclusions

The results of the mycobiota studies revealed the predominance of the genera *Alternaria* and *Fusarium* in the analyzed wheat samples. We noticed that no differences in mycobiota were observed between samples from the seven areas studied, except for *Penicillium* sp. which has a relatively high contamination levels for the area Fès-Meknès, Skhirat-Témara and Rabat-Salé, which is probably due to higher humidity during the year. According to the results of this study it is recommended to monitor the quality of pre and postharvest wheat in the aim of reducing contamination by moulds and mycotoxins at the level of finished product.

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