Mycotoxigenic Fungi in Cereals grains and coffee from the North of Morocco

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Abstract

Agriculture provides the main income for the economy in Mediterranean countries. Morocco, a North African and Mediterranean country, surrounded by the Mediterranean Sea and Atlantic Ocean, is characterized by a hot and humid climate which could probably lead to mold contamination and mycotoxin production especially in coastal areas.

This contribution gives an overview of the contamination by fungal mycoflora of cereals and coffee consumed in the North of Morocco .Some species belonging to the genus *Aspergillus* that are known to be a potential producers of ochratoxin A(OTA), this mycotoxin has been shown to be a nephrotoxic, hepatotoxic, teratogenic and possibly carcinogenic for humans.
The aim of the present work was to know the fungal distribution in some crops collected in northern Morocco, Tangier city. The species of saprophytic fungal were identified including *Aspergillus* and *Penicillium* that contaminate wheat, rice and coffee beans. Studies on fungal DNA detection in 88 fungal samples have been performed. They were identified at the species level, by sequencing the internal transcribed spacer (ITS4, ITS5) of ribosomal DNA (rDNA). This is the first report on the contamination by mycotoxigenic fungi in coffee from Morocco, and also there is no previous work exists on contamination by these fungi in cereals samples from North of Morocco.

Keywords: Fungal mycoflora, *Aspergillus, Penicillum*, rDNA, ITS, cereals, coffee, Morocco.

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1. Introduction

Cereals and derivatives are the basis of the food in Morocco which explains their economic importance. On average, Morocco consumes six million tons of cereals each year. Moreover, cereals contribute to approximately 12% of the agricultural output and Moroccan households

spend 25% of their food expenditure on this kind of product (Zinedine et al., 2009). In addition, by 2020 the Moroccan population will require 8.5 million tons of cereals for the national consumption. Due to drought that the country has endured during the last two decades, cereal yield production has been dramatically reduced in the range of 25–85% (INRA-National Institute of Agronomy Research of Morocco, 2002).

Coffee is consumed widely in the country and has gained a considerable importance by becoming an industry and a staple food. The data from the Exchange Office showed that the volume imported in 2003 was 33,091 tons, with a value of 272 million dirhams (Dh), while in 2007, the volume has not significantly increased 35,644 tons but for a price of 558 million Dh. Due to the considerable and continuous rise in the international coffee prices, the coffee roasting industry in Morocco knows a stagnation (Kherraf., 2010).

Moreover, like other agricultural products, cereals and coffee are naturally favorable to fungal growth that can affect their quality and have several negative consequences: an altered appearance and technological properties of raw materials, the development of fungal infections or allergies, the production and accumulation of mycotoxins which cause economic losses (Bennett et al, 2003).

Fungi exhibit greater species richness than most other organisms and, thus, are of significant environmental and economic importance (Varga et al., 2011; Blackwell, 2011). Recent predictions based on molecular methods have suggested that there are 5.1 million fungal species (O'Brien et al., 2005); however, only approximately 5% of the predicted filamentous fungal species have been described (Hawksworth., 1991).

Fungal growth is one of the main causes of food spoilage. It not only generates great economic losses, but also particularly through the synthesis of mycotoxins.

Some species of the genera *Penicillium* and *Aspergillus* are known to form ochratoxin, but few of them are known to contaminate foods with this mycotoxin. Ochratoxin (OTA) has been detected in food products such as wine, beer, grape juice, dried fruit, meat, figs, coffee and cereals (Abarca et al., 1994; Bayman et al., 2002; Cabanes et al., 2002; Creppy., 2002; Gareis & Scheurer., 2000; Hussein & Brasel., 2001; Stefanaki et al., 2003; Taniwaki et al., 2003; Visconti et al., 2000).

OTA contamination of food is known to be produced only by *A. ochraceus, P. nordicum* and by *P. verrucosum* (Moss., 1996) which affect mainly cereals in different regions of the world. However, some recent studies have clearly shown that in addition to certain species belonging to the black aspergilli, including the *A. niger, A . carbonarius, A. westerdijkiae, A. steynii* and *A. ochraceus* are responsible for the formation of OTA in coffee (Noonim et al., 2008; Taniwaki et al., 2006).

The objectives of this preliminary study were therefore to evaluate the diversity of filamentous fungi and to investigate the presence of potentially mycotoxigenic fungi in samples taken from wheat, rice and coffee available in Tangier, Northwest of Morocco.

2. Materials and methods

2.1. Collection of samples

A total of 104 samples of cereals and 30 samples of coffee commercialized in North of Morocco, Tangier city, were randomly collected from different traditional markets in the city

and were then stored at refrigerator at 4 ° C before being analyzed. The samples included wheat (N = 37), rice (N = 37) and coffee (N = 30).

2.2. Fungal isolation and identification

The samples were examined by the Direct Plating technique described by Pitt *et al.*, (1997). For each sample, one hundred grains was surface disinfected with 0.4% sodium for one minute at room temperature and then were placed on different culture media: Malt Extract Agar medium MEA (Biokar Diagnostics, France), Czapek Dox Agar medium CYA (Scharlauchemie S.A, Spain) and Potato Dextrose Agar medium PDA (Biokar Diagnostics, France) for incubation at 25°C for 5 to 7 days. The identification of fungal strains and the determination of each species of fungi were done using the keys of Klich (2002) for *Aspergillus* sp. and Pitt and Hocking (1997) for *Penicillium* sp. and other genera. This is done by observing both microscopic characteristics of the colonies on various media used as well as the microscopic morphology.

2.3. DNA analysis: Molecular identification

Isolates were purified for molecular identification at the species level. All *Penicillium* sp. strains and some strains of *Aspergillus* sp. were identified.

2.3.1 DNA extraction

Total genomic DNA of isolates was extracted using a modification by Cenis (1992) : 8 day-old mycelium was gently scraped off a PDA Petri dish surface, transferred directly into 1,5 ml Eppendorf tubes containing 300 µl of lysis buffer (200 mM Tris- HCL pH 8,5, 250 mM NaCl ,25 mM EDTA) and homogenized for 3 min using Eppendorf micro pestles (Eppendorf, Hamburg, Germany); 25 µl of 0,5% SDS were then added and tubes were placed at 65°C for 10 min .After addition of 150 µl of 3M sodium acetate, the tubes were placed at – 20°C for 10min, centrifuged at 10,000x g for 30 min and the supernatant transferred to a new tube. The DNA was precipitated by adding an equal volume of isopropanol and left for at least 5 min at room temperature. After 10 min of centrifugation at 10,000x g, the pellet was washed with 70% ethanol, dried at room temperature and resuspended in 100 µl TE-buffer.

2.3.2 PCR assay

The nucleotide sequences separating the large and small ribosomal RNA genes, known as internal transcribed spacer (ITS) regions, are highly variable and often used to distinguish taxonomic groups (Bruns et al., 1991; Hibbett et al., 2005; Geiser et al., 2007). The ITS of the nuclear rDNA was amplified by PCR using the fungus universal specific primers ITS4 (5'-TCCTCCGCTTATTGATATGC -3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG -3') (White et al., 1990). PCR reactions were carried out in a total volume of 50 µl containing 3 U of Taq DNA polymerase (Perkin Elmer), 10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2,10 µM of each primer, 10 mM of each of dNTPs and 10 ng of genomic DNA, PCR was performed in a GeneAmp PCR System 2400 (Perkin Elmer) thermal cycler and cycling conditions were as follows : 94°C for 2 min followed by 35 cycles at 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 7 min .

The amplified products were analyzed by electrophoresis through 1,2 % agarose gel in 0,5 x TBE buffer and 100 bp DNA Ladder (Gibco BRL, Gaithersburg, MD) was used as molecular weight marker. After staining with gel red, the gels were visualized under ultraviolet (UV) photographed by Gel Doc 2000 (Bio-Rad Laboratoires, Hercules, CA).

2.3.3. PCR clean up and DNA sequencing

To obtain a pure DNA, a PCR clean-up using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel product, CA) was done as follows: 200 µl of binding buffer NT1 was mixed with each sample (100 µl of DNA) and to purify the DNA, 700µl of Wash Buffer NT3 was added and centrifuged at 11,000 g for 30 sec, this operation was repeated twice.

The gel and PCR Clean-up NucleoSpin column was placed in a new centrifuge tube with 30µl of elution buffer NE (5Mm Tris-HCl pH 8,5) followed by incubation at room temperature (18-25°C) and centrifuged at 11,000 g for 1 min. DNA was recovered in the pellet.

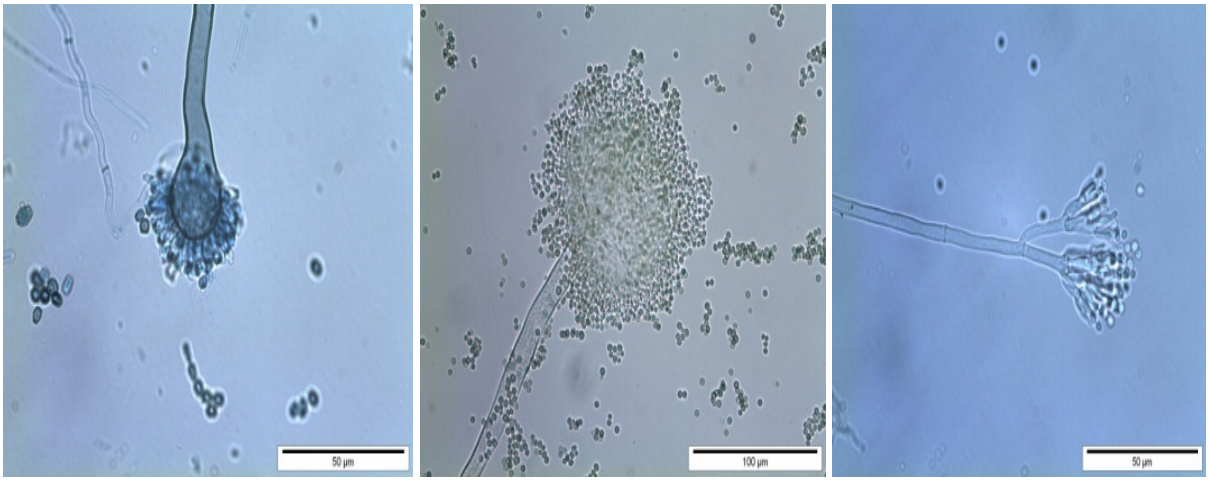
The identity of each strain was verified by morphological characteristics and by comparing the ribosomal DNA (rDNA) sequences with those already deposited in the data at GenBank (NCBI, Bethesda, MD, USA) using BLAST search tool.. The identification of the species was determined based on the best score.

3. Results and Discussion

3.1. Isolation and molecular identification

In this study, the mycoflora is primarily heterogeneous and the average of contaminated samples was often above 86,53%.

From different culture media, various fungi species were isolated as the natural contaminant of cereals (wheat, rice) and coffee in local grains. The isolated genera included *Aspergillus*, *Penicillium*, *Fusarium*, and others like *Alternaria*, *Rhizopus*, *Cladosporium*, and *Mucor*. The most mycotoxigenic fungal species were identified morphologically (Fig.1).



(A) **(B)** **(C)**

Figure 1: microscopic morphology of *A. ochraceus (A), A. flavus (B), P. spinulosum (C).*

According to the Food and Agriculture Organization (FAO), 25% of the World food crops, including many basic foods are affected by mycotoxin producing fungi (Köppen et al., 2010).

Aspergillus and *Penicillium* were obtained from all culture media, except *Fusarium* which was not found on the Czapek Dox medium (Czapek) and isolated in very low frequencies

from the Potato Dextrose agar medium (PDA) and Malt Extract Agar medium (MEA). *Aspergillus* was the most frequently found fungal genus with a high frequency more than 73% in the three culture media, followed by *Penicillium* (15,97%) (Fig.2a).

Potato Dextrose Agar medium (PDA) is the standard medium most widely used for the isolation of fungi in foods. On PDA medium, the incidence of samples contaminated by *Aspergillus* sp. was significantly dominant and from the 488 strains isolated, 387 belong to *Aspergillus* sp. (79.19%), followed by 78 penicillia strains (15, 97%), while the number of *Fusarium* sp. isolates were 3 representing 0,69 % of the total number of isolates, the 4.16% was represented by the other species of fungi (Fig.2a).

Different species were found *A. Niger*, *A. ochraceus*, *A. flavus*, *A. fumigatus* and were predominant while the other species were found at relatively low frequencies different from one medium to another. Figure 2b showed the percentage of occurrence of each species of *Aspergillus* sp. identified in different culture media used (Fig.2b).

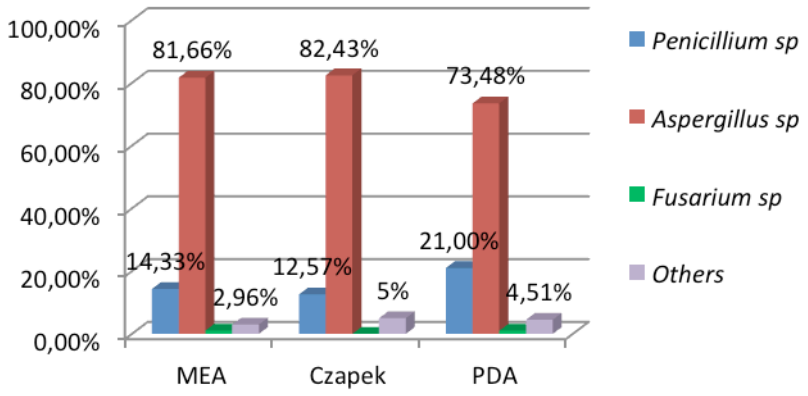
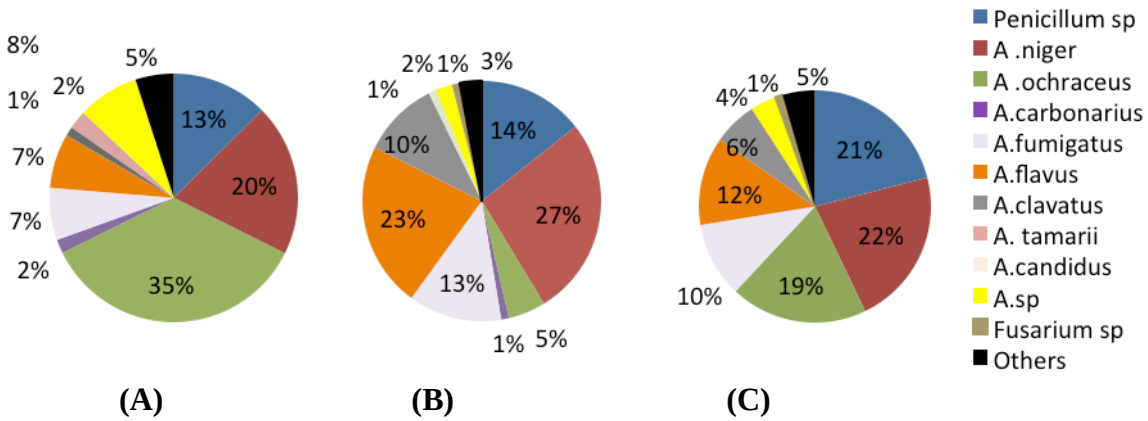


Figure 2a. Frequency of fungi general isolated from different culture media.



(A) **(B)** **(C)**

Figure 2b. The isolation rates of species belonging to *Aspergillus* sp. in different culture media: Czapek medium (A), MEA medium (B), PDA medium (C).

For the molecular identification, it was necessary to select targeted DNA regions having conserved sequences in all organisms that can be used for hybridization of the primers but also a variable sequences specific for a genus, a species or a strain. The difficulty also lies in the fact that fungi such as genera *Aspergillus* and *Penicillium* are highly conserved (91-99% sequence identity) so the difficulty for their differentiation will be increase.

88 strains (all isolated strains of *Penicillium* sp. and 10 strains of *Aspergillus* sp.) were identified genetically to confirm their species.

Penicillium. chrysogenum was the most predominant fungal contaminant with more than 29%, followed by *P. commune* with a value of 21.62%. Others *Pencillium* species such as *P. glabrum*, *P. aurantiogriseum* and *P. admatizoides* were found almost with the same percentage (8.10%). *P. spinulosum* represents a frequency of 5.40% while other species like *P. echinulatum*, *P .tricolor* and *P .rubens* were found with the least frequency (2.70%).

The rest of strains of Penicillium genetically identified represented by several strains (10.81%) are difficult to distinguish from each other, their taxonomy was not fully resolved and that require usually other molecular test to identify precisely their species.

For the 10 Aspergillus strains genetically identified, it was found 3 *A. versicolor*, 2 *A. flavipes*, *3 A. tubingensis and* 2 *Eurotium amstelodami* (Fig.3).

Our results indicated that the diversity of the species is great, it can be explained by the particular climatic conditions especially temperature and humidity of Tangier that had a big influence on the presence of certain mycotoxigenic fungi in foods.

The risk of contamination by mycotoxin is an important food safety concern for cereals and coffee and it's important to note that this contamination pose a serious economic impact worldwide. The economic impact result from lowered productivity reduced feed conversion efficiency and increased disease incidence hence the need to control and to limit mycotoxins by establishment of a specific regulation to ensure food safety.

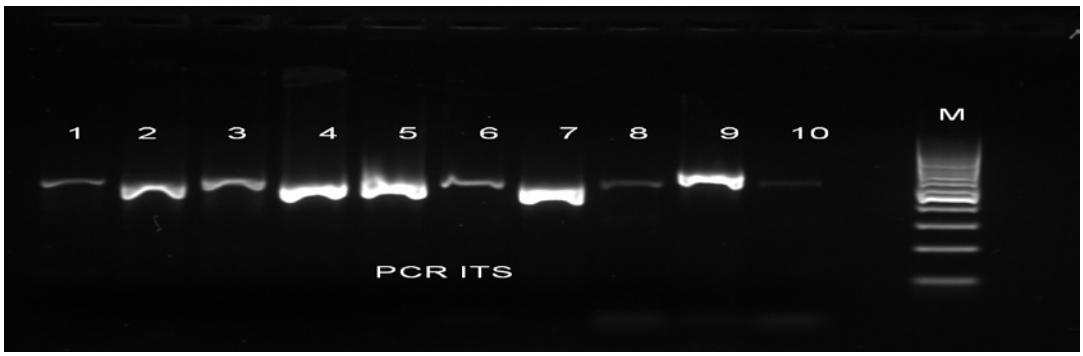


Figure 3: Gel electrophoresis of PCR products of *Aspergillus* sp. amplified by using ITS 4/ITS5. (Molecular weight marker, M.100 bp DNA ladder).

3.2. Cereals

The total fungal contamination and the rates of infection were significantly higher, whatever the cereal analyzed. Wheat was contaminated by different fungal genera and *Aspergillus*

(representing five species) was the most predominant contaminant fungal genus, with over 73% of contaminated grains. Among *Aspergillus* species, *A. ochraceus* (21,50%) was the most frequently found in all tested wheat, followed by *A. flavus* (15%), *A. fumigatus* (13%) and *A. niger* (12,80%). Others *Aspergillus* sp. such as *Aspergillus clavatus*, *candidus*, and *terreus* was also frequently found in wheat.

The genus *Penicillium* has also contaminated approximately 20% of wheat samples. However, others fungal genera such as *Fusarium* sp. and others (*Alternaria*, *Cladosporium*...) were also respectively represented by 2 and 5 % (Fig.4).

For rice grains, the same fungi were found. Besides, approximately 69% of the samples were contaminated by *Aspergillus* sp. The most frequently detected specie was *A. ochraceus* (23,06%). The species *A. niger* (14,10%), *A. fumigatus* (11, 20%) and *A. flavus* (11%) were also detected. *Penicillium* sp. contaminates rice with almost the same value of contamination of wheat (24,33%). *Fusarium sp*. (1%) and others (5,86%) were found but at a significantly lower frequency (Fig.4).

Overall results showed that the *Aspergillus sp*. is the most isolated species in mycoflora as the contaminant of cereal crop. This result is similar to those published by Hajjaji et al., (2005), Tahani et al., (2008), Riba et al, (2008), Gacem et al. (2011), who reported that *Aspergillus* and *Penicillium* were the most frequently isolated genera in the cereals samples analyzed.

Aspergillus identification revealed that *A. ochraceus* reported to be the main specie responsible for OTA production was the most frequent contaminant of rice and wheat.

A. niger was also found with important frequencies. This specie is a very frequent fungal contaminant found worldwide on various substrates such as cereals or coffee bean (Battilani et al., 2006; Leong and al., 2007; Magnoli et al., 2007).). Although this species is not considered as an important mycotoxin producer, it has been shown to produce ochratoxin A, usually at low level (Hajjaji et al., 2006).

These species have already been reported as ochratoxigenic fungi in cereal grains (Czerwiecki et all., 2002; Accensi et all., 2004).

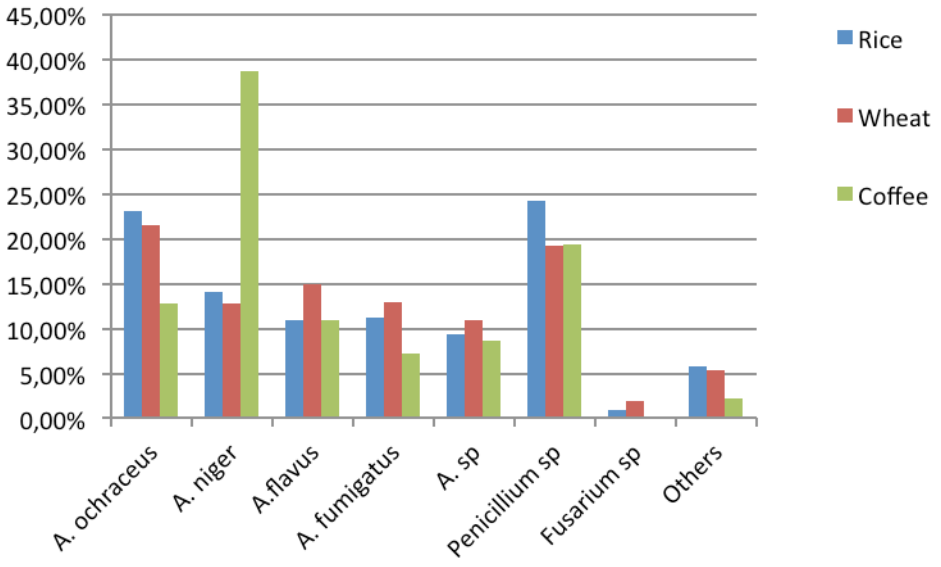


Figure 4: The occurrence of dominant species from rice, wheat and coffee samples.

3.3. Coffee

Like cereals, the coffee beans are subject to contamination by microorganisms during different development stages, from the crop field to storage (Batista et al., 2009).

Coffee beans analyzed by direct plating were contaminated with fungi, including species of Aspergillus, *Penicillium*, *Rhizopus* and yeasts.

The presence of fungi in the coffee beans does not only affect quality in terms of flavor and aroma of the beverage, but also presents a safety risk for the final product, due to the production of toxic secondary metabolites, the mycotoxins, which can be harmful to consumers at certain concentrations (Bennett and Klich, 2003, Vilela et al., 2010).

In our study, a high level of fungal infection was observed (80 %) and the majority of coffee was infected with one or more fungi, the most frequent fungal contaminants belong to *Aspergillus* (78, 33%), *Penicillium* (19, 42%) genus and no *Fusarium* strain was found. A.*niger* (38,68%) was the dominant specie and other fungi commonly isolated included *A.ochraceus* (12,80%), *A. flavus* (11%) and less present *A. fumigatus* (7,18%). These fungi typically coinfected coffee beans with the black Aspergilli. (Fig.4)

These results are in agreement with previous studies that showed that these *Aspergillus* and *Penicillium* were the most important contaminants in coffee (Mislivec et al., 1983; Micco et al., 1989; Bucheli et al., 2000; Pardo et al., 2004; Bokhari., 2007). In addition, many studies revealed that the important toxigenic fungal genera (*Aspergillus* and *Penicillium*) are natural coffee contaminants, and are present from the field to storage during different stages of growing, harvesting, processing, transport and storage (Food., 2006; Nakajima et al., 1997; Silva et al., 2000).

The incidence and severity of infection by *A. niger*, *A. ochraceus* and related species appeared to be less in Moroccan coffee beans (collected after storage) than in Coffee beans from other countries. It can be seen that overall infection rates were low with similarity on the predominance of *Aspergillus* section Nigri (Leong et al., 2007, Urbano et al., 2001; Martins et al., 2003; Taniwaki et al., 2003; Pardo et al., 2004) followed by *A. ochraceus* that was the predominant yellow *Aspergillus* species as a result also found in the work of Taniwaki et al. (2003).

Several researches have been carried out to analyze the presence of ochratoxigenic fungi in coffee (Noonim, Mahakarnchanakul et al., 2008; Silva et al., 2008; Batista et al., 2009, Vilela et al., 2010). The ochratoxin A-producing specie from the genus Aspergillus is A. ochraceus (Taniwaki et al., 2003; Frisvad et al., 2004; Perrone et al.,2007; Batista et al., 2009; Gil-Serna et al., 2011;), A. niger (Taniwaki et al., 2003; Samson et al., 2004; Perrone et al.2007). A. ochraceus is commonly found in coffee and is an important ochratoxin A producer (Suarez-Quiroz et al., 2004b; Batista et al., 2009; Vilela et al., 2011).

In northern Morocco, Tangier city has an outstanding geographical position between Atlantic and Mediterranean coastlines and therefore a climate characterized by high humidity that explain the dominance of saprophytic fungi like species of *Aspergillus* and *Penicillium* known as fungi of storage in foods (Pitt et Miscambe., 1995 ; Tahani et al., 2008 ; Makun et al., 2007). Riba et al., (2008) also showed that poor ventilation coupled with a high temperature promotes the growth of these species.

The growth of *Penicillium* decrease in favor of the growth of *Aspergillus* and the rate of contamination by Penicillium is inversely proportional to the duration of storage, which

explains the dominance of the genus Aspergillus, probably favored by high moisture grain and a long storage period (Tahani et al., 2008).

The prevention and control of mould growth in foods can be accomplished by keeping moisture low, grain fresh or stored at a moisture level less than 14 percent, and equipment clean. Aeration of grain is important to reduce moisture migration, keep the feedstuff dry, and prevent the production of mycotoxin (Jones et al., 1994).

More investigations on the evaluation of OTA levels for different mycotoxigenic strain isolated are necessary to provide data about exposure and to evaluate possible human health risk of OTA.

4. Conclusions

In this research, we revealed a high frequency of the genus Aspergillus, in comparaison with the other genera, both in wheat, rice and coffee.

In Morocco, there is no data on the natural occurrence of mycotoxigenic fungi in coffee, also there is no data on the natural occurrence of mycotoxigenic fungi in cereals in the North of Morocco and little is known about levels of ochratoxin (OTA) in these samples.

Additional studies incorporating analysis of toxin potential are needed to more fully assess the importance of species and contamination of cereals and coffee in order to protect the population from risks associated with mycotoxin contamination.

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