JOURNAL OF FOOD QUALITY & HAZARDS CONTROL
SHAHID SADOUGHI UNIVERSITY OF MEDICAL SCIENCES & HEALTH SERVICES

Journal of Food Quality and Hazards Control 9 (2022) 32-42

Aflatoxins and Ochratoxin A in Red Chili (*Capsicum*) Powder from Tunisia: Co-Occurrence and Fungal Associated Microbiota

S. Lasram *✉, H. Hajri, Z. Hamdi

Laboratory of Molecular Physiology of Plants - Borj-Cedria Biotechnology Center, B.P. 901, Hammam-Lif 2050, Tunisia

HIGHLIGHTS

- Mycotoxins levels in 5 out of 55 samples were above the European Union (EU) limit.
- The highest co-occurrence of mycotoxins was found between aflatoxin B_1 and ochratoxin A.
- *Aspergillus flavus* and *A. niger* were the most toxigenic species identified.
- This study alarms us about the hygienic risk raised by Tunisian consumers.

Article type
Original article

Keywords
Capsicum
Aflatoxins
Ochratoxin A
Mycotoxins
Aspergillus
Tunisia

Article history
Received: 14 Jul 2021
Revised: 6 Dec 2021
Accepted: 21 Dec 2021

Acronyms and abbreviations
AF=Aflatoxin
CFU=Colony Forming Unit
HPLC=High Performance Liquid Chromatography
PCR=Polymerase Chain Reaction
OTA=Ochratoxin A

ABSTRACT

Background: Mycotoxins are produced in foods as a result of mold infection of crops before and after harvest. The aim of this report was to assess, for the first time in Tunisia, the contamination of red chili powder with Aflatoxins (AFs) and Ochratoxin A (OTA) and to identify the associated microbiota.

Methods: Fifty-five samples of red *Capsicum* powder (*Capsicum annuum*) were screened for AFs and OTA and toxigenic fungal species. Mycotoxins were extracted using immunoaffinity columns and quantified by High Performance Liquid Chromatography (HPLC). Dilution method was realized for fungal isolation and confirmed Polymerase Chain Reaction (PCR) analysis. Data were statistically analysed using statistical software (version 5.0).

Results: Mycotoxins levels in 5 out of 55 samples were above the European Union (EU) limit. The highest co-occurrence of mycotoxins was found between AFB_1 and OTA (39/55 samples). *Aspergillus flavus* and *Aspergillus niger* were the most toxigenic species identified. The highest level of molds contamination found in *Capsicum* powder reached 7.91×10^6 Colony Forming Unit (CFU)/g.

Conclusion: The co-occurrence of two important mycotoxins (OTA and AFB_1) observed in this study, alarm us about the hygienic risk raised by Tunisian consumers and raise the need to improve the production process for red *Capsicum* powder in Tunisia.

Introduction

Mycotoxins are natural food and feed contaminants, mostly produced by fungi belonging to *Aspergillus*, *Penicillium*, and *Fusarium* genera. At present, more than 300 to 400 mycotoxins are recognized but the most significant classes from a public health concern are the aflatoxins (AFs), ochratoxin A (OTA), deoxynivalenol, ergot alkaloids, fumonisins, patulin, and zearalenone (Trucksess and Diaz-Amigo, 2011). The Food and Agriculture Organization and the World Health Organization (FAO/WHO) expert committee on food addi-

* Corresponding author (S. Lasram)
✉ E-mail: salma.lasram.cbbc@gmail.com
ORCID ID: https://orcid.org/0000-0003-2257-7743

To cite: Lasram S., Hajri H., Hamdi Z. (2022). Aflatoxins and ochratoxin a in red chili (*Capsicum*) powder from Tunisia: co-occurrence and fungal associated microbiota. *Journal of Food Quality and Hazards Control*. 9: 32-42.

DOI: 10.18502/jfqhc.9.1.9688 Journal website: http://jfqhc.ssu.ac.ir

tives, described the mycotoxins as overwhelming dangers to humans and animals' health through food and feed consumption (JECFA, 2001). AFs and OTA, produced by *Aspergillus* and *Penicillium* molds, are among the most harmful fungal secondary metabolites causing hepatotoxic, carcinogenic, and neurotoxic effects (Reinholds et al., 2016). The AFB_1, listed in Group I carcinogen substances by the International Agency for Research on Cancer (IARC), is the most potent hepatocarcinogen recognized in mammals. Moreover, the IARC considers OTA as possibly carcinogenic to humans under Group 2B carcinogen (IARC, 1993). Moreover, OTA is an immunosuppressive, teratogenic, and nephrotoxic substance (JECFA, 2001).

Mycotoxins can be detected in a wide range of commodities, including cereals, spices, dried fruits, apple products, wine, and coffee (Santos et al., 2010). Among spices, red *Capsicum* powder or chili powder is the second largest consumed spice throughout the world after the black pepper. Chili powder is derived from dry red pepper (*Capsicum*) belonging to the family of Solanacea. It is also called pepperoni pepper powder or red chili pepper.

According to the Food and Agriculture Organization (FAO), in 2016, the worldwide production area for dried *Capsicum* was 1,798,847 ha, with a production of 3,918,159 tons of harvested product per year (Costa et al., 2019). However, due to the fact that they are frequently contaminated with spoilage fungi, the incomes of red pepper producers are compromised. Indeed, chili is mainly cultivated in developing countries characterized with tropical and/or semi tropical climates. High temperature, rainfall, and relative humidity are very favorable to fungal contamination in these growing regions, especially by potentially mycotoxigenic species (Santos et al., 2010). Moreover, they are exposed to be contaminated with spoilage fungi during the production chain due to poor collection conditions, incorrect agricultural practices, and traditional production processes which could cause fungal propagation and exacerbate mycotoxins synthesis.

The European Commission established a legislation for mycotoxin in food, including regulations for AFs in *Capsicum* products with maximum tolerable limits set at 10 µg/kg for total AFs; AFB_1, AFB_2, AFG_1, AFG_2, and 5.0 µg/kg for AFB_1 (EC, 2012). The regulation for ochratoxins also set maximum levels of OTA in spices of 20 µg/kg for *Capsicum* powder and 15 µg/kg for mixtures of chili with other species (EC, 2015).

Dried *Capsicum* derived-products have extensively been reported to be frequently contaminated by mycotoxins in India (Jeswal and Kumar, 2015), Turkey (Özkan et al., 2015; Tosun and Ozden, 2015), Pakistan (Iqbal et al., 2010, 2013), Sri Lanka (Yogendrarajah et al., 2014), Spain (Santos et al., 2010), Thailand (Rotsisen et al., 2016), and Iran (Khazaeli et al., 2017).

In spite of these numerous survey studies, up till now researches dealing with mycotoxins contamination in Tunisian red pepper powder are lacking despite its importance as a national agro-food product and its large consumption through the local cuisine. In Tunisia, pepper cultivars are mainly represented by *C. annuum* L. species with a number of chili pepper landraces cultivated throughout the country (Lahbib et al., 2013). The Cap Bon, Kairouan, and Sahel regions in Tunisia are the main zones of pepper cultivation; mainly composed of traditional populations of *C. annuum* spp. Three local accessions namely 'Piment Sesseb', 'M'sarreh', and 'Rouge Long' populations had the highest total capsaicinoid contents in pepper fruit and are largely cultivated for pepper fruit production in season crops transformed to a spice usually called "red pepper powder" (Ben Mansour-Gueddes et al., 2010). According to Costa et al. (2019), amongst types of mycotoxins detected in *Capsicum* pepper derivatives, AFs and OTA are among the most frequent contaminants. Thus, the aim of this report was to assess, for the first time in Tunisia, the contamination of red chili powder with AFs and OTA and to identify the associated microbiota.

Materials and methods

Sampling

A total of 55 samples of red *Capsicum* powder were randomly collected during 2019 from several retailers in different regions of Tunisia including the main producing area with different climatic conditions (Bizerte/ North 2; Tunis and Siliana/ North-ouest 8; Beja, Mateur and Jendouba/ North-est 7; Cap-Bon peninsula/ North-East 21; Sahel/ East 5; Sfax and Sidi Bouzid/ Center 10; Gabes/ South 2). Samples (500 g of each) were placed in sterile plastic bags and stored at 4 °C until analysis.

Mycological tests

Fungal genera were isolated and enumerated from red *Capsicum* powder by dilution method as described by Pitt and Hocking (2009). Ten g of each sample were added to 90 ml of sterile peptone solution (1%) in 500 ml Erlenmeyer flask and homogenized with an electric shaker for 30 min. Ten-fold serial dilutions were, then, prepared and 100 µl aliquots of each dilution were plated, in triplicate, on Dichloran Rose Bengal Chloramphenicol Agar (DRBC) plates. Petri dishes were incubated at 27 °C for 7 days, then, all visible fungal colonies were counted and expressed as Colony Forming Unit (CFU)/g for each sample.

[DOI: 10.18502/jfqhc.9.1.9688]

Primary characterization of the strains, at genera level, was carried out by morphological and microscopical observations according to Pitt and Hocking (2009). Fungal colonies belonging to main potentially ochratoxigenic and aflatoxigenic species (*Penicillium* spp. and *Aspergillus* sections *Flavi*, *Circumdati*, and *Nigri*) were recorded and the number of CFU/g was calculated for each sample. Representative colonies of each different subgenus were plated on Potato Dextrose Agar (PDA) and pure cultures were obtained through monosporic isolation method. For identification at species level, strains were cultured in malt extract agar (MEA) and czapek yeast extract (CYA) media and were incubated 7 days at 27 °C. Taxonomic characterization was performed according to Abarca et al. (2004), Klich (2002), and Pitt and Hocking (2009). *Aspergillus* section *Flavi* strains were, also, cultured and incubated 7 days at 25 ºC in *A. flavus* and *parasiticus* agar (AFPA) which is a selective media for *A. flavus* and *A. parasiticus* identification (Rodrigues et al., 2007).

Molecular analysis

To confirm the morphological identification, a selected number of mycotoxigenic strains were subject to molecular characterization. Genomic DNA of each strain was obtained using a cetyl trimethylammonium bromide (CTAB) extraction method (Šarkanj et al., 2018) and was tested for suitability for Polymerase Chain Reaction (PCR) with universal ITS_1 and ITS_4 primers. PCR assays were carried out using species specific primers (Sardiñas et al., 2011); the primers FLA_1 (5′-GTAGGGTTCCTAGCGAGCC-3′) and FLA_2 (5′-GGAAAAAGATTGATTTGCGTTC-3′) for *A. flavus* (González-Salgado et al., 2008), and ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and NIG (5′-CCGGAGAGAGGGGACGGC-3′) for *A. niger* aggregate (González-Salgado et al., 2005). Amplification reactions were carried out in a volume of 25 µl containing 1 µl of template DNA, 4 µl of each primer (5 µM), 5 µl of 5x PCR buffer, 1 µl of $MgCl_2$ (25 mM), 0.5 µl of deoxynucleoside triphosphates (dNTPs) (10 mM), and 0.2 µl of *Taq* DNA polymerase (5 Unit/µl). The PCR amplification protocol for *A. flavus* was as follows: 1 cycle of 5 min at 95 °C, 26 cycles of 30 s at 95 °C, 30 s at 56 °C, 45 s at 72 °C and, finally, 1 cycle of 5 min at 72 °C. For *A. niger*, the protocol was as follows: 1 cycle of 4 min 30 s at 95 °C, 25 cycles of 30 s at 95 °C, 25 s at 66 °C, 40 s at 72 °C, and finally 1 cycle of 5 min at 72 °C.

PCR products were separated in 2% agarose ethidium bromide gels in 1x TAE buffer (Tris-acetate 40 mM and (ethylenediaminetetraaceticacid) EDTA 1.0 mM). A 100 bp DNA ladder was used as molecular size marker and *A. flavus* (GenBank: MW465763.1) and *A. niger* (GenBank: MW604719.1) were used as representative strains.

Toxigenic ability of the isolates

OTA and AFs production of the isolates was determined as described by Bragulat et al. (2001) in CYA extract agar medium (Sucrose 30 g; $NaNO_3$ 2 g; K_2HPO_4 1 g; $MgSO_4+H_2O$ 0.5 g; KCl 0.5 g; $FeSO_4+7H_2O$ 0.01 g; Agar 15 g; Distilled H_2O 1 L). The pH of the test media was adjusted at 5.5. The agar plates were inoculated at the middle and incubated for 7 days at 25±2 °C. At the end of the incubation period, three plugs (6 mm diameter) were removed from the middle, outer, and inner area of the colony. Plugs were put in a vial with 1 ml of methanol as High Performance Liquid Chromatography (HPLC) grade. Sixty min later, the methanolic extracts were shaken, passed through 0.45 µm filters (MillexR SLHV 013NK, Millipore, Bedford, Massachusetts, USA), and stored at temperature 4 °C until the HPLC analysis.

Determination of AFs and OTA

The mycotoxins analysis in *Capsicum* powder was performed according to the European commission's regulations No. 657/2002 for official control of mycotoxins in foodstuffs. For the analysis of AFs (AB_1, AB_2, AG_1, and AG_2) and OTA, the extraction and cleaning-up of the samples were performed using AflaStarTM and OchraStarTM immunoaffinity columns (IACs), respectively, according to the manufacturer's instructions. Twenty-five g of chili powder were added to 100 ml mixture of methanol:water (60:40, v/v) for AFs and methanol:water (80:20, v/v) for OTA; then, blended at high speed for 3 min. The samples were centrifuged at 5,000 rpm during 10 min and filtered through Whatman filter paper. Eight ml of the supernatant were recovered then diluted with 16 and 24 ml of phosphate buffer saline solution for AFB_1 and OTA analysis, respectively. The diluted extract was passed through the IAC and eluted at 1-2 drops/s. Then, the column was washed with 20 ml of deionized water and the fixed mycotoxins were eluted with 2 ml (2x1 ml) of methanol HPLC grade for AFs or a solution of methanol:acetic acid (98:2, v/v) for OTA. The methanolic extract was dried with a SpeedVac concentrator and re-suspended in 0.5 ml of HPLC grade methanol.

HPLC analyses

Detection and quantification of AFs and OTA were performed by HPLC with fluorescence detection. The HPLC apparatus (KNAUER, Germany) was equipped with a C18 column (Waters Spherisorb 5 µm, ODS2,

[DOI: 10.18502/jfqhc.9.1.9688]

4.6×250 mm) and a pre-column of 10×4 mm placed in a thermostat at 40 °C. The mobile phase was constituted with acetonitrile/water/acetic acid (57:41:2, v/v/v) for OTA and acetonitrile/water/methanol (50:20:30, v/v/v) for AFs and run at the flow rate of 1.0 ml/min (injection volume 25 µl). For AFs, a post-column derivatization by Iodine (2%) was realized. The detection was carried out by a fluorescence detector (Waters 474, Milford, Massachusetts, USA) at λ_{exc} 365 nm and λ_{em} 440 nm for AFs; and at λ_{exc} 330 nm and λ_{em} 460 nm for OTA.

Calibration curves were set up by six OTA standard solutions (0.1, 5, 10, 50, 100, and 500 ng/ml) and six AFs standard solutions (2.5, 5, 10, 20, 50, and 100 ng/ml for each AF) in HPLC-grade methanol. Retention times for AFG_2, AFG_1, AFB_2, and AFB_1 were 6.3, 7.4, 8.7, 10.1, and 16.9 min, respectively, and 4.7 min for OTA. The Limit of Detection (LOD)/Limit of Quantification (LOQ) were established at 0.02/0.06, 0.02/0.06, 0.1/0.3, 0.05/0.15 ng/g for AFB_1, AFB_2, AFG_1, AFG_2, respectively, and 0.02/0.06 ng/g for OTA. Recovery experiments were performed by spiking AF-free chili samples with mycotoxins levels of 0.5 and 2 ng/g of each AF and OTA and spiking was done in triplicate. The mean recovery values of AFB_1, AFB_2, AFG_1, AFG_2, and OTA were 94.5, 96.3, 84.3, 91.1, and 97.4%, respectively.

Statistical analyses

Owing to the non-normality of mycotoxin amount results, the non-parametric Spearman correlation coefficients were used to identify correlations among mycotoxins in samples using STATISTICA software (version 5.0, StatSoft, Inc., Tulsa, OK, USA).

Results

Fungal contamination

The results of the mycological analyses showed that the majority of chili samples (30/55) had a total microbial density ranged between 10^3 and 10^4 CFU/g. The highest level of molds contamination found in *Capsicum* powder reached 7.91×10^6 CFU/g (Table 1).

Toxigenic ability of strains

Fungal species isolated in our study are described as being capable to synthetize mycotoxins, in this context, *Aspergillus* section *Flavi* isolates were assessed for their aflatoxigenic potential and *Aspergillus* section *Nigri* and *Penicillium* molds were tested for the OTA production ability. Quantitative differences in AFs and OTA *in vitro* production between *Aspergillus* isolates is presented in Table 2. Molds were classified in four groups according to their toxigenic potential. A total of 63 isolates belonging to *Aspergillus* section *Nigri* and 25 *Penicillium* spp. were tested. Only 14% (22/63) were found to produce OTA *in vitro* with 50% of positives strains producing OTA at levels superior to 1,000 ng/g. However, no *Penicillium* isolate was able to produce OTA.

According to their capacity to produce the four major types of AFs (AFB_1, AFB_2, AFG_1, and AFG_2) on synthetic medium (CYA), the tested strains were classified in different chemotype: Type I for strains producing only AFB_1, Type II for strains producing AFB_1 and AFB_2 and Type III for strains producing AFG_2. It should be noted that no isolate was able to produce AFG_1 at detectable level. The obtained results revealed that out of the 46 AFs producing molds, 37 belongs to chemotype II, 9 are the chemotype I, and only 2 isolates are the chemotype III (Table 2). The majority of isolates (39%) were able to produce AFB_1 at level superior to 1,000 ng/g.

The majority of toxigenic isolates belonging to *Aspergillus* section *Flavi* presented macro and microscopic features of *A. flavus*, while ochratoxigenic *Aspergillus* section *Nigri* isolates behoved to *A. niger* aggregate group. Molecular identification was also performed on selected strains from *Aspergillus* section *Flavi* (n=13) and *Aspergillus* section *Nigri* (n=7) and the analysis supported that all of them belonged to *A. flavus* and *A. niger* species, respectively.

Mycotoxins occurrence

As indicated in Table 3, the most prevalent mycotoxin was AFB_1 (90%) followed by OTA (80%). However, the OTA concentrations were superior with values ranged between 0.5-35.23 ng/g. For AFB_1, the concentration ranged between 0.1-27.07 µg/kg in positive samples. Furthermore, analysis showed that contamination with AFB_2 is also substantial (56%) but with lower amounts, ranging from 0.32 to 1.62 µg/kg. Overall, only 3 and 2 samples exceed the maximum levels that have been established for AFs and OTA in spices, respectively.

Moreover, the results showed that 26/55 samples contained AFB_1, AFB_2, and OTA simultaneously, 39/55 samples contained AFB_1 and OTA and 36/55 were contaminated with both AFB_1 and AFB_2. In order to search for a possible correlation between the contamination of samples with mycotoxins, non-parametric Spearman correlation coefficients (r_s) were calculated among mycotoxins in samples (AFB_1/AFB_2; AFB_1/OTA, and AFB_2/OTA). Values of r_s equal to 0.1638 (p=0.23), -0.18665 (p=0.17), and -0.18665 (p=0.17) were obtained between the variables AFB_1/AFB_2; AFB_1/OTA, and AFB_2/OTA amounts, respectively. This shows that there is non-significant (p>0.05) and negligible correlations between the concentration of the analyzed mycotoxins.

[DOI: 10.18502/jfqhc.9.1.9688]

Table 1: Fungal contamination (Colony Forming Unit (CFU)/g) of red *Capsicum* powder

	Count (CFU/g)			
	$<10^3$	10^3-10^4	10^4-10^5	$>10^5$
Section *Nigri*	6	12	15	0
Section *Flavi*	6	12	22	6
Penicillium spp.	3	3	2	1
Total microbiota	2	14	30	9

Table 2: Toxigenic ability of *Aspergillus* section *Flavi* and *Nigri* isolates from red *Capsicum* powder in Czapek Yeast Extract Agar (CYA) medium

Mycotoxin (n [a])	Range (ng/g)	Number isolate (%)	Mean (ng/g)±SD
AFB_1 (46)	<10	9 (20)	4.4±2.6
	10-100	6 (13)	31.6±24.1
	100-1,000	13 (28)	307.9±157.9
	>1,000	18 (39)	6444.2±5640.9
AFB_2 (37)	<10	7 (19)	4.4±2.8
	10-100	22 (59)	33.0±12.3
	100-1,000	8 (22)	211.7±116.4
	>1,000	0	-
AFG_1 (0)	<10	0	-
	10-100	0	-
	100-1,000	0	-
	>1,000	0	-
AFG_2 (2)	<10	1 (50)	13.2
	10-100	1 (50)	3.1
	100-1,000	0	-
	>1,000	0	-
OTA (14)	<10	2 (14)	1.1±0.06
	10-100	2 (14)	43.7±1.5
	100-1,000	3 (21)	433.5±266.9
	>1,000	7 (50)	2319±135.3

[a] n=number of toxigenic strains/total number of tested strains

AF=Aflatoxin; OTA=Ochratoxin A

Table 3: Occurrence and level of aflatoxins and ochratoxin A (OTA) in red *Capsicum* powder samples

Mycotoxin	Positive, n (%) [a]	Frequency distribution of samples (µg/kg), n (%) [a]			Contamination (µg/kg)	
		[LOQ [b]-5]	[5-10]	≥ 10	Range	Mean±SD
OTA	44 (80)	21 (38)	17 (31)	6 (11)	0.5-35.23	6.4±4.14
AFB_1	50 (90)	47 (94)	-	3 (6)	0.1-27.07	2.72±2.22
AFB_2	31 (56)	31 (100)	-	-	0.32-1.62	0.59±0.2
AFG_1	9 (16)	9 (100)	-	-	0.19-1.66	0.74±0.42
AFG_2	6 (11)	6 (100)	-	-	0.20-1.13	0.63±0.22

[a] n represents the number of samples and the data in parenthesis shows the percentage of these samples.
[b] LOQ: Limit of Quantification (0.06, 0.06, 0.3, 0.15 ng/g for AFB_1, AFB_2, AFG_1, AFG_2, respectively and 0.06 ng/g for OTA)
AF=Aflatoxin

[DOI: 10.18502/jfqhc.9.1.9688]

Discussion

Capsicum powder, as a dehydrated product, can represent a favorable environment to the development of mycotoxigenic fungi owing to the post-harvest practices and the environmental storage conditions. Various studies have shown the contamination of dry red pepper and its derivatives with mycotoxins, in particular with AFs and ochratoxins (Costa et al., 2019; Yogendrarajah et al., 2014). However, report on the occurrence of mycotoxins and identity of toxigenic fungi in red *Capsicum* powder from Tunisia is missing. On this context, this work is an effort to investigate the contamination level of this product with OTA and AFs and to identify the associated toxigenic fungi.

The International Commission on Microbiological Specifications for Foods set up a maximum limit of 10^4 CFU of molds and yeasts/g of spices (ICMSF, 1986). Our results revealed that analyzed chili samples are highly contaminated with molds with 71% of the samples having a fungal load exceeding the maximum limits set in international food regulations. Previous studies have reported high level of microbial contamination in *Capsicum* powder. Melo González et al. (2017) investigated the microbiological quality of Argentinian paprika and reported fungal counts ranging between 2×10^2 and 1.9×10^5 CFU/g exceeding in several samples the maximum limits set in international food regulations. Hashem and Alamri (2010) compared the contamination of common spices in Saudi Arabia markets with fungi and assembled them into three groups according to their affinity to be infected with molds. The authors classified red pepper powder into the first groups including spices which produced >1,000 CFU/g and considered this group to have a high affinity to contamination. However, fungal load of analyzed sample could vary according to the isolation medium. Indeed, Santos et al. (2010) reported 2.3×10^4 mean total CFU/g of paprika in MEA and 3.8×10^2 CFU/g of paprika in DG18 medium.

The study of fungal load in spices is of importance since the quality of the foodstuffs deteriorates consequently of mold spoilage. Furthermore, the presence of some genus able, potentially, of producing mycotoxins presents a health risk to the consumer. In the present work, among *Aspergillus* species, section *Flavi* fungi were the most occurring toxigenic species, followed by *Aspergillus* section *Nigri* moulds. *Penicillium* spp. fungi were not frequently isolated. The high presence of *Aspergillus* strains in samples is predictable. Indeed, in the drying, storage, packaging, and transportation steps, *Aspergillus* and *Penicillium* species are the major spoilage fungi in *Capsicum* by-products due to their xerophilic characteristics which allow them to get a competitive advantage, in low water activity conditions, compared to other fungal pathogens (Costa et al., 2019). The dominance of the two sub-genera *Flavi* and *Nigri* in the pepper powder microbiota was notified by different studies. Chuaysrinule et al. (2020) examined the presence of ochratoxigenic and aflatoxigenic fungi in dried chili from Thailand and reported that the most recurrent fungus was *Aspergillus* section *Flavi* (46.6%), followed by *Aspergillus* section *Nigri* (34%), *Penicillium* (7.6%), and *Aspergillus* section *Circumdati* (2.2%). It's worth to notice that, in our study, no *Aspergillus* section *Circumdati* were isolated from chili samples knowing that this group includes species with high OTA potential. Our results corroborate with the findings of Almela et al. (2007) who studied the occurrence of OTA in paprika elaborated from peppers grown in several countries (Peru, Brazil, Zimbabwe, and Spain). The author reported that no *Aspergillus* section *Circumdati* moulds was isolated from Spanish paprika samples. However, a high percentage of fungi belonging to this group was found in chili from Peru, with 30.76% of ochratoxigenic strains identified as *A. ochraceus*. However, Santos et al. (2010) reported the presence of *Aspergillus* section *Circumdati*, *Nigri*, and *Flavi* isolates in chili and paprika from Spain. Nevertheless, *Aspergillus* section *Nigri* had the highest relative density of potentially toxigenic *Aspergillus* in the samples (62.47%) and *Aspergillus* section *Circumdati* had the lowest one (16%). Among the fungal isolates belonging to the *Aspergillus* section *Nigri* and *Penicillium* spp. tested for OTA production, only 14% proved toxigenic with 50% of positive strains producing OTA at levels above 1,000 ng/g. Thus, although a small percentage of black aspergilli having an OTA producing ability, some isolates were highly toxigenic. Our results did not corroborate with the findings of Almela et al. (2007) who tested the ochratoxigenic potential of thirty isolates of aspergilli section *Nigri* isolated from Spanish paprika, but none of them was able to produce OTA and only one strain (1.69%) of black *Aspergillus* isolated from Peruvian samples was ochratoxigenic.

The ability of *Aspergillus* spp. to produce ochratoxin in admitted since a long time. The OTA is produced by *P. verrucosum* in temperate or cold climates and by *Aspergillus* species in warmer climates. Within *Aspergillus* section *Nigri* group (black aspergilli), *A. carbonarius* and *A. niger* are the main fungal species producing OTA (Pitt and Hocking, 2009).

Otherwise, 72% of the *Aspergillus* section *Flavi* isolates were found aflatoxigenic. The high frequency of aflatoxigenic fungi in the *A. Flavi* group isolated from *Capsicum* powder has been commonly observed (Costa et al., 2019). Our results are consistent with a previous report by Singh and Cotty (2017), who analyzed chillies from markets in Nigeria (n =55) and the United States (n=169). The authors observed that out of the 205

[DOI: 10.18502/jfqhc.9.1.9688]

isolates *Aspergillus* section *Flavi* isolates from chili, over 70% of isolates produced AFs.

Moreover, our study showed that the majority of isolates from *A. Flavi* group (39%) were able to produce AFB_1 at level superior to 1,000 ng/g, however, 59% of AFB_2 producing isolates had a lower toxigenic potential inferior to100 ng/g. It has to be noted that the nine most producing AFB_1 isolates are those belonging to chemotype I (producing only AFB_1). The herein presented results demonstrate that the majority of toxigenic *Aspergillus* section *Flavi* strains had the ability to produce simultaneously the AFB_1 and AFB_2 on synthetic medium, with higher amounts for the AFB_1 which is considered as the most recurrent and potent carcinogens in foods among the AF group (JECFA, 2001). High amounts of AFB_1 produced in culture media alarms us about the potential of the isolates to produce such amounts of this toxin in the *Capsicum* product. The low proportion of *Aspergillus* section *Flavi* strains producing AFs G was emphasized in different works. Chuaysrinule et al. (2020) reported that 96.9% of the 96 AF-producing *Aspergillus* section *Flavi* isolated from Thai dried chili were AFB producers and only 3 isolates produced both AFB and AFG (3.1%). A similar low frequency of AFB and AFG production amongst fungus has been found for strains isolated from dried red chili in the United States and Nigeria (Singh and Cotty, 2017).

The section *Flavi* group contains the major economically significant AF-producing fungi, *A. flavus* (Klich, 2007). Initially, it was admitted that strains of *Aspergillus* section *Flavi* producing AFB_1 and AFB_2 belong to *A. flavus* species and that the strains producing AFB and AFG were *A. parasiticus* (Varga et al., 2011). However, the taxonomy of the aflatoxigenic species of *Aspergillus* section *Flavi* has been evolving continuously for ten years and several new species have been described since 2011. Thus, numerous species were described as able to produce the B and G type AFs such as *A. nomius, A. minisclerotigenes, A. luteovirescens, A. sergii, A. aflatoxiformans, A. novoparasiticus, A. austwickii, A. cerealis, A. pipericola, A. mottae,* and *A. pseudocaelatus* (Frisvad et al., 2019).

Thereby, in the present study, we only focused on the characterization of the strains with the most toxigenic ability to identify species involved in the contamination of our product. All these species were firstly characterized by micro and macro morphological characters and then confirmed by specific PCR assays.

In the present work, the most toxigenic strains from *Aspergillus* section *Flavi* (n=13) and *Aspergillus* section *Nigri* (n=7) belonged to *A. flavus* and *A. niger* species, respectively. Characterized *A. flavus* isolates belong to chemotypes I and II, producing only AFB group which is predictable because of the inability of this specie, except in rare cases, to produce type G AFs. In addition, despite the discovery of new aflatoxinogenic species, *A. flavus sensu stricto* remains the most species producing AF type B (Frisvad et al., 2019). In our study, the main ochratoxigenic black *Aspergillus* species, *A. carbonarius* was not existing in chili samples. In accordance with our findings, *A. niger* and *A. flavus* species, among others being part of sections *Nigri* and *Flavi*, have been found to be the main fungal contaminants in *Capsicum* derivative products and were largely being related to the occurrence of mycotoxins in such food products (Costa et al., 2019). Singh and Cotty (2017) reported that the *A. flavus* strains were the dominant species of *Aspergillus* section *Flavi* (84%) in dry chilies. Sardiñas et al. (2011) has performed a molecular detection of potentially mycotoxigenic *Aspergillus* species in *Capsicum* powder by a highly sensitive PCR-based method. The results showed that the most frequent aspergilli were *A. niger* aggregate (67.7%), followed by *A. flavus* (49.5%). *A. carbonarius*, *A. parasiticus*, and *A. steynii* were isolated at lower incidence (1.1%). Garcia et al. (2018) found only *A. flavus* and *A. niger* complex among the OTA and AFs producing fungi in Pepperoni pepper. Melo González et al. (2017) evaluated the microbiological quality of paprika produced in Catamarca (Argentina) and concluded that *A. flavus*, a possible producer of AFs type B and cyclopiazonic acid, was moderately frequent, whereas *A. parasiticus*, generally an important producer of AFs type B and G, was found in only one sample. The author also reported that *A. niger* was the most frequently isolated ochratoxigenic fungi. However, the presence, in dried chili, of other *Aspergillus* toxigenic species was described by Chuaysrinule et al. (2020). The latter reported the presence of highly ochratoxigenic strains of *A. carbonarius* and *Aspergillus alliaceus*, but with low frequency. Furthermore, the tested *A. niger* and *A. ochraceus* group strains exhibited no OTA production.

The presence of toxigenic isolates of *A. flavus* and *A. niger* in samples could lead to the contamination of *Capsicum* powder with mycotoxin. Our results showed that the most widespread mycotoxin in our samples was AFB_1 (90%) followed by OTA (80%) with higher mean concentration for OTA. Otherwise, an important co-occurrence of mycotoxin was observed with 26/55 samples contained AFB_1, AFB_2, and OTA simultaneously, 39/55 samples contained AFB_1 and OTA and 36/55 were contaminated with both AFB_1 and AFB_2.

The important co-occurrence of such hazardous toxins, found in this study, could pose a serious health threat to the consumer. Fifty over 55 samples contained one or more of these toxins with higher contamination. These results are in accordance with data presented by Abass (2019) who revealed that the co-occurrence of AFs and OTA was highly detected (35%) in African countries and

[DOI: 10.18502/jfqhc.9.1.9688]

was comparatively less in the European Region (24%). The toxicity of mycotoxins mixtures cannot be only assessed based on their individual toxicities. However, multi-exposure may result in antagonist, additive, or synergic effects that could cause more harmful effects on human health (Smith et al., 2016). The European Commission Regulations fixed the maximum levels of individual mycotoxins in *Capsicum* powder to 5 µg/kg for the AFB_1; 10 µg/kg for the sum of AFs (B_1, B_2, G_1, and G_2) and 20 µg/kg for the OTA (EC, 2012, 2015). In our study, despites the high prevalence of AFs and OTA, only 3 (5.4%) and 2 (3.6%) samples, respectively, exceed the maximum levels that have been established for these mycotoxins in spices. However, these positive samples were significantly above the thresholds established by European Commission (13.938, 14.359, and 27.078 µg/kg for AFB_1; 20.88 and 35.23 µg/kg for OTA).

Globally, our data showed clearly high contamination of *Capsicum* powder analyzed in the present study, mainly, with OTA and AFB_1 confirming the previous report on the occurrence of these toxins in *Capsicum* derivatives all over the world (Costa et al., 2019). Moreover, in our study, we can assume that *A. flavus* and *A. niger* species are the main ones responsible for the contamination of *Capsicum* powder with AFBs and OTA, respectively.

According to Costa et al. (2019), regarding processed pepper products (e.g., crushed pepper, powdered pepper, and paprika), they are more susceptible to AF contamination than fresh fruit. Compared to our results, higher contamination with AFB_1 was observed in chili powder from Pakistan. Iqbal et al. (2010) has analyzed ground chili samples for the presence of AFB_1 and reported a very high mean concentration in positive samples of 32.20±9.15 µg/kg. Almost 86.4% of ground chillies were contaminated with AFB_1 above the European Union (EU) permissible level. In more recent study, Iqbal et al. (2013) reported again high contamination percentages with AFB1 of chili powder 60% (mean 12.75±0.70 µg/kg; max 84.6 µg/kg) and crushed chili 64% (mean 13.90±0.98 µg/kg, max 90.6 µg/kg) collected from open markets in Pakistan. OTA was detected, in a lesser degree, in 38% of crushed chili (mean 16.9±2.1 µg/kg, max 54.3 µg/kg) and 38% of chili powder (mean 21.4±1.9 µg/kg, max. 64.5 µg/kg). However, these amounts are significantly higher than OTA concentrations found in our study.

The same, Reddy et al. (2001) evaluated the contamination of chili powders and chili pods by AFB_1 collected from India. The Authors found that 59% of chili samples were contaminated with AFB_1 and 18% contained the toxin at non-permissible levels. The highest level of AFB_1 contamination was found in pepper pods (969 µg/kg). In agreement with our results, lower contamination levels with mycotoxins were reported by several researches conducted in Mediterranean countries. From Turkey, Aydin et al. (2007) have reported that 68% of powdered red pepper samples were found positive with AFB_1 and levels were found to be higher than the legal limits in 18% of samples (max 40.9 µg/kg). Ozbey and Kabak (2012) has analyzed 22 red chili samples from Turkey and found that 63.6% of red chili powder contained AFs at detectable levels and three red chili powders were found above the EU regulatory limit for AFB_1. OTA was found in 54.5% of red chili powder with mean of 24.65 µg/kg. From Spain, Santos et al. (2010) reported that 59% of 64 paprika samples (AFB1 max 2.66 µg/kg; AFs max 7.25 µg/kg) and 40% of 34 chili samples (AFB_1 max 2.49 µg/kg; AFs max 4.66 µg/kg) were contaminated with AFs. None of the samples had AFs levels higher than the allowable limit. Pepper powders were more contaminated with OTA; 98% for paprika (max 281 µg/kg) and 100% for chili (max. 44.6 µg/kg). Percentages of 37% and 44% of the paprika and chili samples, respectively, had OTA concentrations above the limits which are higher than the reported in our study. Similarly, to our results, very low levels of AFG_1 and AFG_2 were reported in chili and paprika samples from Spain. Hernández Hierro et al. (2008) analyzed 21 paprika samples from Spain for AF and OTA contamination, of which 13 samples contained both AF (range 0.7-4.5 µg/kg) and OTA (range 0.7-73.8 µg/kg). OTA was detected in 67% of the paprika samples with a mean level of 11.9 µg/kg. Our results corroborate with the findings of Yogendrarajah et al. (2014), who studied the co-occurrence of multiple mycotoxins in dry chili samples from different origins. The author reported a higher co-occurrence of AFB_1 and OTA in 329 samples (23%) than AFB_1 and AFB_2 co-occurrence (14%). Some studies have reported high incidence of AFs but low mean concentration. Shundo et al. (2009) from Brazil has pointed out that 82.9% of paprika samples were contaminated with AFs, and AFB_1 was detected in 61.4% at concentrations ranging from 0.5 to 7.3 µg/kg with a mean of 3.4 µg/kg. The author also found that 85.7% of samples were OTA positive at amount ranged from 0.24 to 97.2 µg/kg (mean 7.0 µg/kg). Almela et al. (2007) reported low mean levels of 3-4 µg/kg of OTA in red paprika commercial samples from Spain. The author found great variances in OTA content in paprika samples and suggested a relationship with the climatic conditions of the geographic origin of the samples and with cultural and technical practices in pepper manipulation.

Likewise for the contamination by AFs which seems to be very variable between studies carried out in the same country, despite a certain tendency which results in a greater contamination in the countries of South-East Asia, probably due to the tropical climate which characterizes these countries and to the traditional methods of

[DOI: 10.18502/jfqhc.9.1.9688]

drying and processing chili peppers. Indeed, according to Costa et al. (2020) peppers are among the spices that are most prone to mold contamination, in particular by potentially mycotoxigenic species. The author highlights the critical factors favouring fungal development and mycotoxin synthesis all through the pepper powder production chain such as excessive irrigation and fertilizer application, late harvest, sun drying (processing time, exposure to soil insects, and spoilage fungi), conditions of transport (hygiene, humidity, and temperature control), and packaging (rehydration, packaging material). The drying phase seems to be the most critical in the *Capsicum* powder production chain. In fact, in the main producing countries (Asia, Africa, and, Central/South America), pepper drying under the sun is the most widespread practice, involving prolonged phases at variable temperature and humidity, offering the optimal conditions for mycotoxins production.

In our work, the determination of non-parametric Spearman correlation coefficients among mycotoxins in samples (AFB_1/AFB_2, AFB_1/OTA, and AFB_2/OTA) showed that there are no correlations between the concentrations of the analyzed mycotoxins. This is in disagreement with Santos et al. (2010) who reported that the presence of OTA was correlated with the presence of AFB_1 and total AFs in paprika and chili samples, concluding that the fungal species responsible for the synthesis of these mycotoxins need alike growing conditions. However, this explanation is not always correct because even if, overall, the favorable environmental conditions for the growth of ochratoxigenic and aflatoxigenic fungi are close, according to different authors (Lasram et al., 2016). The optimal conditions for the production of AFB_1 and OTA, respectively, by *A. flavus* and *A. niger* are different.

Conclusion

This study showed that chili powder from Tunisian markets is frequently contaminated with OTA and AFs, although, the levels of contamination were not alarming with few samples exceeding the authorized limits. However, the high rate of co-occurrence of OTA with AFB_1 and AFB_1 with AFB_2 indicated that these mycotoxins might be implicated in a wide range of synergistic and additive interactions. Daily exposure to these mycotoxins mixtures through consumption of food containing *Capsicum* powder might contribute to exceeding the tolerable daily intakes of these mycotoxins causing a variety of adverse health effects for the Tunisian consumer. Thus, careful Hazard Analysis and Critical Control Point (HACCP) techniques during fresh pepper production and the following phases of drying, transportation, elaboration, and storage are essential to prevent the risk of mycotoxin contamination of *Capsicum* powder.

Author contributions

S.L. performed the mycotoxin analysis, the fungi isolation, and wrote the manuscript; H.H. performed the molecular characterization of the isolates and contributed to data analysis; Z.H. designed and supervised the study. All authors read and approved the revised manuscript.

Conflicts of interest

All the authors declared that this is no conflict of interest in the study.

Acknowledgements

This research was financially supported by the Tunisian Ministry of Higher Education and Scientific Research.

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