



Growth Characterization of Mycotoxigenic Molds Isolated from Bread Flour Consumed in Algeria

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ABSTRACT

Background: Cereals, including soft wheat, are widely consumed in Algeria because of the high demand for bread. Given the low water availability in baking flour, molds are most likely to grow and some are toxigenic, which may affect the quality of this product. The objective of this work was to determine and evaluate the fungal flora of bakery flour as well as to highlight its mycotoxigenic power and growth ability.

Methods: A total of 241 samples were collected from different places in the western Algerian region. The enumeration was carried out according to Algerian standard regulation. The obtained fungi were identified basing on microscopic identification. After identification the *Aspergillus* and *Penicillium* genera were tested for the mycotoxins production on Y.E.S medium following by TLC revelation. As for the growth study, the growth was tested on PDA medium than adjusted by Barany's model.

Result: Mold enumeration revealed an overall contamination rate of 97.93% with concentrations (cfu/g) ranging from 1 to 15 log cfu/g. The identification of fungal genera revealed the presence of eleven genera of molds, namely, *Penicillium* sp. (69.29%), *Cladosporium* sp. (56.85%), *Microsporum* sp. (51.04%), *Aspergillus* sp. (34.85%), *Auerobasidium* sp. (24.07%), *Exopiala* sp. (21.58%), *Mucor* sp. (17.43%), *Trichothecium* sp. (9.13%), *Trichoderma* sp. (8.71%), *Streptomyces* sp. (2.07%) and *Trichosporon* sp. (0.41%). Only the genera *Aspergillus* and *Penicillium* showed a mycotoxins production reveal by the TLC method on YES media. The simulation of growth during storage at the bakery showed a remarkable increase that exceeded the threshold (4 log) for each genus and a significant increase ($P < 0.001$). These results are of great industrial interest for improving the quality and reducing the risk linked to the consumption of the product.

Key words: *Aspergillus*, Flour, Mycotoxins, *Penicillium*.

INTRODUCTION

Cereals are more widely consumed worldwide, have significant nutritional power and constitute the basis of the human diet. In Algeria, among the most consumed cereals in several forms, soft wheat is the most consumed. Flour is the main product made from soft wheat and is mainly used for the preparation of bread. In Algeria, 49 million baguettes are consumed every day, making them the leading bread-consuming country in the world (Belhocine, 2010).

In most cases, cereal production is ensured by a single harvest during the year, which requires storage, which can sometimes occur under poorly controlled conditions and can affect the presence and growth of several mycotoxigenic fungi during the storage of soft wheat until bread is produced (Chukwudi *et al.*, 2021; Dutta *et al.*, 2018; Okioma *et al.*, 2021; Lavkor *et al.*, 2019).

Wheat flour is characterized by lower (0.8 - 0.87) water activity (Feillet, 2000) and is produced without any processing for removing microorganisms. The fungi could survive at low a_w , as reported by Dooletkedieva (2010); Graves et Hasseltine (1966); Deligeorgakis *et al.* (2023) and Kichu *et al.* (2019). With the climate change observed in recent years, fungal contamination is certainly increasing, as reported by Rezazadeh *et al.* (2013) for

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Aspergillus flavus, *Aspergillus ochraceus*, *Aspergillus versicolor*, *Penicillium citrinum*, *Penicillium citreoviride*, *Penicillium cyclopium*, *Penicillium martensii*, *Penicillium patulum* and *Penicillium pubertum*. Some of these species are toxigenic, such as *Aspergillus flavus*, *Aspergillus parasiticus*, *Penicillium expansum* and *Penicillium crustosum* (Afssa, 2006).

These mycotoxigenic fungi can produce toxic secondary metabolites called mycotoxins (Awuchi *et al.*, 2021). Mycotoxins are hazardous for consumer health and can cause great economic losses (Bhat and Vasanthi, 2003). Due to the heat stability of mycotoxins, their residue can persist in bread after heating and can be eaten by consumers.

Even if the dose is nontoxic, repeated exposure to a low dose of mycotoxins in bread is likely to cause chronic poisoning. In the long-term, mycotoxins cause harmful effects on the cardiovascular, respiratory and digestive systems, as well as on the nervous system and the urinary system (Omotayo *et al.*, 2019). It also induces immuno-suppressive, genotoxic, carcinogenic and teratogenic mutagenic effects (Bennet and Klich, 2003; Afssa, 2006). The objective of our work was to study the contamination of soft wheat flour used for bread making in western Algeria by mycotoxigenic fungi and to model its growth ability.

MATERIALS AND METHODS

The current research was carried out at University of Tlemcen from November 2020 to July 2023.

Sampling

A total of 241 samples of flour used for bread making in bakeries in different regions of western Algeria (Tlemcen, Ain Temouchent and Sidi Belabbes) were taken for research on mycotoxinogenic fungi. The sampling zones were selected using the areal method. It consists of randomly surrounding areas on the map. Then, each area was visited to spot bakeries and removed from the dough mixer, which was filled only with flour. A mass of 50 g of flour was taken and then placed in a sterile bag. All samples were coded as shown in Table 1.

Mycological analyses

The flour analysis was carried out following the procedure described by the Algerian standard of the Official Journal No. 52/2015, which describes the horizontal method for the enumeration of yeasts and molds by counting the colonies in the products, including those for which the water activity is less than or equal to 0.95 (a_w).

Sample preparation

The procedure consisted of suspending a mass of 1 g of each sample in 9 mL of TSE. This mixture constitutes the first dilution, which will be used to prepare the range of dilutions. The mixture was rigorously shaken between each sample.

Counting of mold colonies

A volume of 100 μ L of each dilution was spread on the surface of a Petri dish containing a Dichloran agar medium supplemented with 18% glycerol (enzymatic digest of casein: 5 g, bacterial agar: 15 g, chloramphenicol: 0.1 g, D-glucose: 10 g, magnesium sulfate: 0.5, potassium

dihydrogenphosphate: 1 g, dichloromethane (2,6 dichloro-4-nitro-aniline): 0.002 g, glycerol: 220 g), a solution of trace elements (ZnSO_4 , CuSO_4) and an oxytetracycline antibiotic at a rate of 50 mg/L to prevent the development of bacteria (Hocking and Pitt, 1980). The inoculated Petri dishes were incubated at $25^\circ\text{C} \pm 1^\circ\text{C}$ for five to seven days.

Purification of mold colonies

The single-spore method described by Noman *et al.* (2018) was used to purify the isolates. Each colony mold developed on the previous medium (dichloran agar) was removed and then transplanted individually using a sterile platinum loop in the center of a Petri dish containing PDA medium, after which the cultures were incubated for five days at 25°C .

Identification of mold species

The identification of the mold genera of the obtained isolates was based only on microscopic observation of the colonies from culture on PDA media. Macroscopic observation proved difficult due to variability in the macroscopic aspects of colonies. In fact, for some fungi strains, the colony changes the color aspect according to the medium composition, others one affected by the light and time incubation. Thus, the identification was based on microscopic observation of isolates is based essentially on the morphological appearance and characteristics of the thallus and spores. Pitt and Hocking (1997) guide were used to identified the fungi.

The smear preparation consisted of adhering a fraction of the colony using a piece of tape and then sticking it on a slide containing a drop of mounting liquid (cotton blue). Microscopic examination was carried out using an optical microscope at $\times 10$, $\times 40$ and $\times 100$ magnification.

The use of microscopic identification does not prevent the macroscopic aspect from being ignored. isolates (the color, size and texture of the colonies, the color of the reverse, the appearance of the thallus and the presence of a pigment).

Study of mycotoxin production

Detection of Mycotoxins by Thin-Layer Chromatography was carried out according to the procedure described by Scott *et al.* (1970). Thirty isolates of mold were inoculated with a colony fragment in vials containing 50 mL of yeast extract sucrose (YES) broth enriched with Tri B vitamins (B1, B6, B12). The cultures were incubated at 25°C for 14 days.

After incubation, the cultures were filtered using Wattman No. 01 type filter paper. Then, the obtained filtrate was mixed with 100 mL of chloroform, stirred for 10 min and then decanted using a separatory funnel. The operation was repeated by successively adding 50 mL and 30 mL of chloroform to the recovered aqueous phase, after which the chloroform phase was concentrated by evaporation under vacuum using a rotary evaporator. After drying, the extract was returned to a glass vial enclosed by parafilm for analysis by thin layer chromatography.

Estimation of the growth parameters of mold isolates

For each tested mold isolate, a 2 mm diameter fragment was inoculated in the center of Petri dishes containing PDA media. During incubation, the colony diameter was measured with a ruler (Baranyi *et al.*, 1993). From the start of growth, the colony diameter was 2 mm. The growth was monitored twice daily for one week.

The primary growth model of Baranyi *et al.* (1993) was used to fit the growth of the tested isolate. The growth parameters (growth rate μ °C: $\mu_{\max}$ and lag phase : λ) of each mold isolate were estimated by equation 1.

$$y = y_0 + \mu_{\max} A - \ln \left(\frac{1 + [\exp(\mu_{\max} A) - 1]}{\exp(y_{\max} - y_0)} \right) \quad \dots 1$$

$$A = t + \left(\frac{1}{\mu_{\max}} \right) \ln [\exp(-\mu_{\max} t) + \exp(-\mu_{\max} \lambda) - \exp(-\mu_{\max} t - \mu_{\max} \lambda)] \quad \dots 2$$

Where,

y_0 = Initial colony radius at time 0.

y = Colony radius at time (t : days)

λ (days) = Latency time at t (days).

A = A variable, depending the t , $\mu_{\max}$ and λ , used only to simplified the equation.

Statistical analysis

The ANOVA analysis was carried out using (SPSS) computer program, ver. 22.0 and the significance among the samples was calculated at $P \leq 0.05$.

RESULTS AND DISCUSSION

Prevalence of contamination

Mycological analyses of all flour samples taken from different bakeries in the regions of Ain Témouchent, Tlemcen and Sidi Belabbès revealed the presence of molds. These results show a dominance of molds, which reached an overall contamination rate of 97.93%. These results are similar to the results obtained by Graves and Hesseltine (1966); Moreau (1970); Halt *et al.* (2004) and Al-Defiery and Merjan (2015).

The region of Ain Témouchent was the most contaminated region, followed by the regions of Tlemcen and Sidi Belabbès, which were less contaminated, with contamination rates of 100%, 98.25% and 93.33%, respectively. The ANOVA analysis showed a significant difference

between two regions of Ain Témouchent and Tlemcen against Sidi Belabbès. This difference may be related to high moisture of Ain Témouchent and Tlemcen compared with Sidi Belabbès (Saïfouni and Bellatreche, 2020). Mohammed *et al.* (2015) reported a correlation accounting of molds and moisture content in wheat flour samples. According also to Saric *et al.* (2008), cereals and cereal products can be contaminated with molds in any phase of a processing cycle: in fields, during harvest, storage, processing, transport and over a period between production and consumption.

Mold count

The enumeration results obtained by this study are shown in Table 1. This indicates a total average mold load of 5.71 log (cfu/g). This concentration exceeds the threshold "M = 4 log" required by the Algerian regulations of the decree of July 2, 2017.

The results obtained show a variation in concentration between the studied regions of 3.35 log (cfu/g) for samples collected from Ain Témouchent, 9.32 log (cfu/g) for Tlemcen and 7.16 log (cfu/g) for Sidi belabbès. ANOVA revealed a significant difference between the different analyzed brands. This variability is probably due in particular to the brand processing and quality of the raw material and flour used as well as the processing, preparation and storage conditions, such as the environment, humidity, time and temperature.

The contamination results obtained in this study are greater than the contamination (1.5 to 1.7 log cfu/g), (<2.3 log cfu/g) and (<2 log cfu/g) reported by Al-Defiery and Merjan (2015), Tahani *et al.* (2008) and Zebiri (2020), respectively. Only the results for samples from the Ain Témouchent region are in accordance with the results (0 - 4.2 log cfu/g) reported by Halt *et al.* (2004). The fungal concentrations (cfu) and contamination frequencies are summarized in Table 1.

The high concentrations of mould contamination could be result of inadequate sanitary measures performed during especially transport and storage of raw material, processing.

Mold identification

The results of macroscopic examinations of the colonies are characterized by variability in appearance depending on the type of mold. The main results are illustrated in Table 2. Indeed, as an example of an isolate identified as

Table 1: Distribution of fungi contamination in wheat flour according to the region.

Regions	Number of samples	Frequency (%)	Fungal concentration: log (cfu/g)			
			Minimum	Maximum	Average	Median
Ain Temouchent	124	100	1	12.89	3.35	2.30
Tlemcen	57	98.25	0	15.22	9.32	9.45
Sidi bel abbes	60	93.33	0	13.35	7.16	7.53
Total	241	97.93	0	15.22	5.71	5.20
ANOVA test	0.001					

Penicillium sp. (Fig 1), on PDA media, colonies of a bluish green color with a white outline, downy to powdery, cottony, velvety, with brown granules, were observed.

Microscopic examination of the pure isolate was carried out by observation at $\times 40$ magnification. The results reported in Table 2 reveal 11 different genera of mold: *Penicillium* sp., *Cladosporium* sp., *Microsporum* sp., *Aspergillus* sp., *Aureobasidium* sp., *Exophiala* sp., *Mucor* sp., *Trichothecium* sp., *Trichoderma* sp., *Streptomyces* sp. and *Trichosporon* sp.

Among the genera identified in this study, *Penicillium* sp., *Aspergillus* sp., *Cladosporium* sp. and *Mucor* sp. have also been identified by several researchers (Halt et al., 2004; Rezazadeh et al., 2013; Al-Defiery and Merjan, 2015). Other researchers have isolated only *Aspergillus* sp. and *Penicillium* sp. (Tahani et al., 2008). According to Pelhate (1982) and Berthier and Valla (1998), the genera *Penicillium*

sp. and *Aspergillus* sp. are considered storage contaminants.

In this study, the list of genes identified was not exhaustive. That is, the absence of unidentified genera does not negate their presence. Indeed, the presence and prevalence are significantly linked to the number of samples tested, the quantity and number of samples, the sampling method and the analysis method and procedure.

The different identified genera had markedly variable contamination frequencies from all tested samples; the most frequent were *Penicillium* spp. (69.29%), followed by *Cladosporium* spp. (56.85%), *Microsporum* spp. (51.04%) and *Aspergillus* spp. (34.85%). In addition, other less important genera were also present, namely, *Aureobasidium* sp. (24.07%), *Exophiala* sp. (21.58%), *Mucor* sp. (17.43%), *Trichothecium* sp. (9.13%), *Trichoderma* sp. (8.71%), *Streptomyces* sp. (2.07%) and *Trichosporon* (0.41%).

Table 2: Distribution of fungi genus in analyses flour.

Genera	Contamination frequency (%)	Concentrations log (cfu/g)		
		Minimum	Maximum	Average
<i>Penicillium</i>	69.29	1.00	3.34	2.17
<i>Cladosporium</i>	56.85	1.00	3.28	2.40
<i>Microsporum</i>	51.04	1.00	3.79	2.61
<i>Aspergillus</i>	34.85	1.00	3.66	2.00
<i>Aureobasidium</i>	24.07	1.30	3.26	2.06
<i>Exophiala</i>	21.58	1.00	3.20	2.14
<i>Mucor</i>	17.43	1.00	2.90	2.06
<i>Trichothecium</i>	9.13	1.00	3.08	2.18
<i>Trichoderma</i>	8.71	1.60	2.48	2.06
<i>Streptomyces</i>	2.07	1.00	1.85	1.26
<i>Trichosporon</i>	0.41	2.00	2.00	2.00

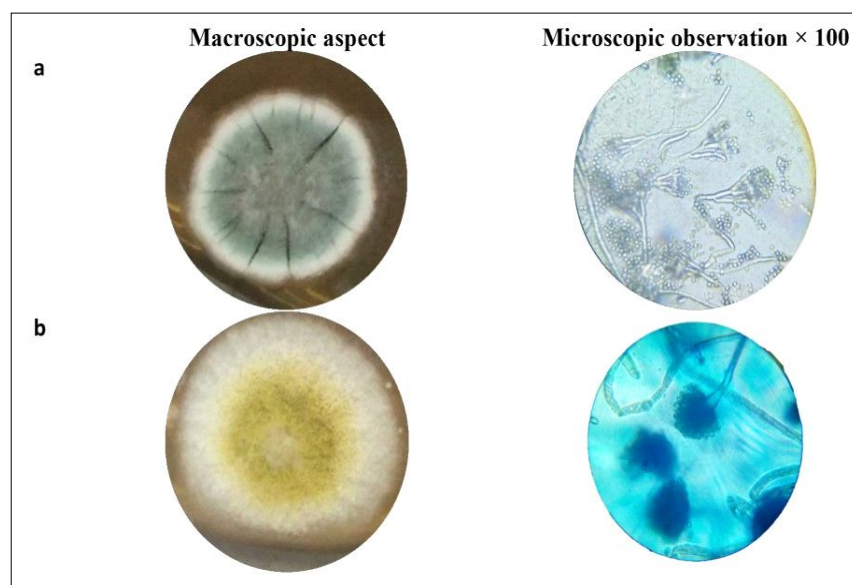


Fig 1: Macroscopic and microscopic ($\times 100$) observation of two example moulds. a: *Penicillium* sp. isolate PF5 and b: *Aspergillus* sp. isolate AF3.

Table 3: Growth parameters of isolated fungi isolated from flour, estimated at a challenge temperature of 25°C.

Genera	Number of tested isolates	Mycotoxins production (%) by TLC	D ₀ (cm)	D _{max} (cm)	lag (λ) (day)	μ_{max} (day ⁻¹)
<i>Penicillium</i> spp.	49	98	0,17±0,11	2,92±0,84	0,079±0,08	0,805±0,92
<i>Aspergillus</i> spp.	31	97	0,17±0,13	28,80±46,65	0,43±0,64	0,397±0,292

Revelation of mycotoxin-producing strains by TLC

Thin-layer chromatographic separation makes it possible to confirm the results previously obtained on CEA media. Chromatographic separation *via* thin-layer TLC makes it possible to separate the extracts from the secondary metabolites found at the wheat flour substrate level by organic solvents and the detection of the extracts under UV radiation at a wavelength equal to 365 nm allows the detection of blue and green stains, which confirms the presence of mycotoxins. The toxin that appears with blue fluorescence is likely aflatoxin B1, which is produced mainly by *Aspergillus*. The *Penicillium* genus likely produces ochratoxin A, which emits green fluorescence under UV light. Betina (1985), Atanda *et al.* (2013) were used this technique to detect mycotoxins exhibiting blue fluorescence and blue-green fluorescence under UV light (365 nm) during the identification of mycotoxin production by the tested isolates.

Thin layer chromatography reveals the production of mycotoxins in the YES medium. The same previously tested strains were tested on YES media. Additionally, only the genera *Aspergillus* spp. and *Penicillium* sp. showed mycotoxin production.

Indeed, *Aspergillus* and *Penicillium* are storage fungi and are known to be producers of mycotoxins (Lozada, 1995). Several studies, such as Rahman *et al.* (2015) and Shareef *et al.* (2010), have also reported the production of mycotoxins by these two genera. This technique is quick, easy to perform and allows for the simultaneous processing of multiple samples (Marin *et al.*, 2013).

Otherwise, several works reported the presence of mycotoxins in wheat flours that ranged from 0.7–74.9% and their average contamination levels in wheat flours (0.2–57.6 $\mu\text{g kg}^{-1}$) (Zhou *et al.*, 2022). These mycotoxins are produced by mould.

Modeling of mold growth

Applying predictive microbiology to quantify the cfu concentration is difficult (Gibson and Hocking, 1997) because filamentous fungi are not unicellular. They form a mycelium whose weight, except at the early stage of growth, does not increase exponentially. Additionally, it is not possible to divide the mycelium into individual cells. Therefore, the colony forming unit (CFU) quantification method can only be used to enumerate spores (Vindeløv and Arneborg, 2002).

In general, the kinetic models used are those based on measuring colony diameter, a simple technique for

obtaining data. The growth of molds on solid substrates under optimal conditions and in the absence of limiting factors generally follows a model consisting of a latency time (λ_{ToC}) and a linear growth phase (to estimate μ_{ToC}). Under unfavorable conditions, a stationary phase can appear when fungi stop growing (Gibson *et al.*, 1994 and Vo *et al.*, 2024).

The growth kinetics were adjusted using the Baranyi *et al.* (1993) model as reported by Dantigny *et al.* (2005). It shows a good fit, with R^2 values between 0.90 and 1. The isolates tested showed different growth capacities, as indicated by the latency time (λ_{ToC} : day) and growth rate (μ_{ToC} : day⁻¹). Indeed, the latency times and μ_{ToC} range between [0.01 to 2 days] and [0.17 to 8 days⁻¹], respectively. The results show that the growth parameters depend on the strain (Table 3). Indeed, variability has been observed within the same genera. Furthermore, the mycotoxigenic molds (*Aspergillus* spp. and *Penicillium* spp.) presented intermediate latency times (λ_{ToC}) and growth rates (μ_{ToC}) compared to those of the other isolates tested (Table 3).

The growth kinetics obeys first-order kinetics characteristic of microbial growth, similar to that of bacteria, having different growth phases: latency time (λ_{ToC}), exponential phase (μ_{ToC}) and stationary phase.

Throughout the food chain, from the field to the consumer's plate, molds are likely to develop and produce toxins, especially if ecological conditions (humidity and temperature) are favorable. Contamination of food or seeds can occur before or during the storage of baking flour. Most toxic molds grow in foods with low water activity by producing mycotoxins.

The results showed an ability to grow in wheat flour. Heenan *et al.* (1998) reported a growth of some *Aspergillus* sp., *Penicillium* sp. and *Fusarium* in wheat flour samples after three months of storage period. Among them there are *A. ochraceus*, *P. viridicatum*, *P. cyclopium*, *P. verrucosum*, *A. niger*, *A. mellis* and *A. carbonarius* (Heenan *et al.*, 1998).

Others authors reported a growth of this fungi in bread with mycotoxins contamination (Ollinger *et al.*, 2024). the consumption of small amount of mycotoxins daily and frequently could accumulates in liver the uptake of these causing serious damage and liver cancer (Halt *et al.*, 2004).

CONCLUSION

Fungal contamination of baking flour is inevitable and the flour can grow during product storage. Among these fungi, some genera can produce mycotoxins that are involved in consumer health damage. The main results revealed the

presence of a large broad range of genera, including mycobacterium fungi (*Penicillium* and *Aspergillus*), that report a production of mycotoxins laboratory medium. Repeated consumption of these mycotoxins can cause damage to public health. To this end, this work has outlined a risk assessment as a perspective.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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