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TOXIGENIC MOLDS CONTAMINANTS IN SUDANESE POULTRY FEED AND THEIR POTENTIAL BIOCONTROL

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Abstract

This study focuses on Penicillium and similar other mold contaminants found in poultry feed sourced from Sudan. Such fungi were found in sorghum kernels, shelled peanuts, wheat bran and peanut meal from seven Sudanese regions. These fungi were isolated and characterized based on their microscopic features and colony morphology on Dicloran Rose Bengal Chloramphenicol Agar (DRBC), Potato Dextrose Agar (PDA), and Pentachloronitrobenzene - Rose Bengal - Yeast extract - Sucrose Agar (PRYES). To evaluate their mycotoxigenic potential, chromatographic and genetic approaches were used. Fungal extracts were evaluated for mycotoxins content by Thin-Layer Chromatography (TLC). Structural and regulatory genes such as nor-1, ver-1, omt-A, aflR, ota.nps and patN, involved in mycotoxins production were analyzed by classical PCR technology. The strains were also identified by sequencing the ITS1 – 5,8S – ITS2 region. To inhibit the growth of Penicillium isolate certain biocontrol agents were used, revealing good antifungal potential. These findings provide valuable insights into the nature of Penicillium contaminants in Sudanese poultry feed, providing fundamental knowledge for further research on managing mycotoxigenic contaminants and feed safety.

Key words*: Biocontrol, Mycotoxin, Penicillium, Poultry feed, Thin-Layer Chromatography (TLC), PCR, sequencing.*

INTRODUCTION

Mycotoxin contamination, in poultry feed threatens the health of animals and the safety of our food (Haque et al., 2020). Among the fungi known to produce mycotoxins, *Alternaria, Aspergillus, Fusarium,* and *Penicillium* species have been identified as contaminants in different agricultural products (Perrone & Susca, 2017).
Revealing the mycotoxigenic potential of mold contaminants is essential to understand the health impact of these substances and to ensure the safety of poultry products and protect consumer health (Hocquette et al., 2005; Bou et al., 2009).
In the current study, the prevalence of blue-like mold contaminants and their mycotoxigenic potential was investigated in poultry feed sourced from Sudan. Microbiologic, molecular and chromatographic approaches were used to examine the prevalence of blue mold contaminants and their toxigenic metabolites in four types of feed. Certain microbiologic strategies to suppress *Penicillium* spp. growth were also examined in order to find a managing solution to reduce the prevalence of mycotoxigenic contaminants to enhance feed safety strategies.

MATERIALS AND METHODS

Fungal isolation from poultry feed

Four types of Soudanese poultry feed were microbiologic analyzed to evaluate the presence of blue mold contaminants: sorghum kernels (A samples), shelled peanuts (B samples), wheat bran (C samples), and peanut meal (D samples). The samples were collected from different locations in Sudan.
The sorghum kernels and shelled peanut samples were ground as powder, in laboratory conditions, using sterile mortar and pestle.
For the microbiological analysis, 10 g of each powder sample were infused in 90 ml sterile distilled water, and vigorously shaken. Serial dilutions were prepared till 10^{-3} in aseptic conditions. To evaluate the fungal load 0.1 ml of suspension was spread (in triplicate) on

Dichloran Rose-Bengal Chloramphenicol Agar (DRBC medium; Carl Roth GmbH+Co.KG, Karlsruhe, Germany) (Beuchat, 1992; 1995). All plates were incubated at room temperature for 4 days to allow fungal growth. Visual analysis was periodically made to enumerate the fungal load. Blue-like colonies were selected and purified on Rose-Bengal Chloramphenicol Agar (RBC; Merck KCaA, Darmstadt, Germany), and subcultured on Potato Dextrose Agar (PDA; VWR Chemicals, Louvain, Belgium).

Microscopic and macroscopic analysis

Isolated fungal cultures were visually analyzed for macroscopic characterization. Slide cultures were also prepared on PDA for microscopic analysis. When needed, methylene blue staining was used to highlight the conidiophores and conidia architecture.

Molecular analysis for fungal identification

Fresh fungal biomass was used for genomic DNA extraction. Commercial ZR Fungal/ Bacterial MiniPrep™ kit (ZymoResearch, SUA) was used, according to the manufacturer instructions, for DNA purification. The resulting DNA was used as template for the amplification of the ITS1-5.8S-ITS4 region. In the PCR was performed in 50 µl reaction volume, containing 1X Green Buffer with $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each ITS1 and ITS4 primers (Table 1), 0.2 U of DreamTaq DNA Polymerase (Thermo-Scientific, Baltics, UAB, Vilnius, Lithuania), and ~10 ng of template DNA, all mixed in sterile MilliQ water. The amplification program included an initial denaturation at 94°C of 4 min, followed by 35 cycles of 1 min at 94°C for denaturation, 1 min at 45°C for primers' annealing, and 2 min at 72°C for elongation, followed by a final elongation at 72°C for 10 min. The PCR products were revealed through gel electrophoresis, using 1% agarose with ethidium bromide, in 0.5X TBE buffer. The electrophoretic profiles were analyzed under UV light, compared to a 100 bp DNA ladder (ThermoScientific, Baltics, UAB, Vilnius, Lithuania), using a BioDoc-It Imaging System (Ultra-Violet Products Ltd., Upland, CA, USA). The PCR products were purified and sequencing by Sanger dideoxy sequencing method, at CeMIA (Cellular and Molecular Immunological Applications, Greece). The BioEdit biological sequence alignment editor was used to assembly the forward and reverse sequencing results. For taxonomic identification the online NBLAST software was used and the partial sequences of the ITS1-5.8S-ITS4 region were compared to similar sequences found in the National Center for Biotechnology Information (NCBI) database.

To evaluate the mycotoxigenic potential of the isolated fungi, chromatographic and genetic approaches were used.

Molecular analysis for mycotoxin encoding genes

The fungal isolates were screened for five structural and regulatory genes involved in mycotoxins production, *aflR, omt, otanps, nor-1,* and *ver-1*. The PCR was performed in 25 µl reaction volume, according to the previously mentioned protocol. The amplification programs slightly varied, depending on the optimal annealing temperature for each tested primer set (Table 1).

The PCR was prepared in 3 steps. The first step was performed for initial denaturation of template DNA, at 94°C for 4 min. The second step had 30 cycles of 1 min at 94°C for denaturation, 1 min at proper primers' annealing temperature, and 2 min at 72°C for elongation. The third step was carried out for the final elongation at 72°C for 7 min.

The PCR products were migrated in agarose gel electrophoresis and visualized in UV light, as previously mentioned.

Thin-Layer Chromatography (TLC).

Fungal strains were grown for 6 days at 28°C, on Pentachloronitrobenzene - Rose Bengal - Yeast extract - Sucrose Agar (PRYES), containing 150 g/L sucrose, 20 g/L yeast extract, 0.1 g/L pentachloronitrobenzene, 0.025 g/L rose Bengal, 0.05 g/L chloramphenicol, 20 g/L agar, and a pH of 5.6±0.2. Four mycelial plugs, each of 6 mm diameter, were collected to perform the mycotoxin extraction. Grounded samples were immersed in 1 ml chloroform, vigorously vortexed, and kept for 2 days at 4°C to improve mycotoxins extraction. Samples liquid phase was then collected by centrifugation, and let for total evaporation. The crude extracts were stored by refrigeration until evaluation. For the TLC analysis, the crude

extracts were resuspended in 25 µl of chloroform, and 10 µl of each sample were spotted on thin-layer silica gel-coated glass plates. An ochratoxin A standard solution (10 µg/ml), provided from Trilogy Analytical Laboratory Inc., was used as 2 µl/spot.

Table 1. Primers sets and their nucleotide sequences

Target DNA region / gene	Primer code	Sequence 5' to 3'	Annealing temperature	Product size (bp)	Reference
ITS1-5.8S-ITS2	ITS1	TCCGTAGGTGAACCTGCGG	45°C	variable	White et al (1990)
	ITS4	TCCTCCGCTTATTGATATGC			
nor-1 or *aflD* gene encoding for norsolorinic acid	NorF	ACCGCTACGCCGGCACTCTCGGCAC	65°C	400	Abdel-Hadi et al. (2010)
	NorR	GTTGGCCGCCAGCTTCGACACTCCG			
ver-1 or *aflM* gene encoding for versicolorin	VerF	GCCGCAGGCCGCGGAGAAAGTGGT	65°C	537	Abdel-Hadi et al. (2010)
	VerR	GGGGATATACTCCCGCGACACAGC			
omt-A or *aflP* gene a structural aflatoxin gene	OmtF	GTGGACGGACCTAGTCCGACATCAC	65°C	797	Abdel-Hadi et al. (2010)
	OmtR	GTCGGCGCCACGCACTGGGTTGGGG			
aflR gene an aflatoxin regulatory factor	AflrF	TATCTCCCCCCGGGCATCTCCCGG	65°C	1032	Abdel-Hadi et al. (2010)
	AflrR	CCGTCAGACAGCCACTGGACACGG			
ota.nps gene encoding for ochratoxin	otanps F	AGTCTTCGCTGGGTGCTTCC	58°C	750	Rashmi et al. (2013)
	otanps R	CAGCACTTTTCCCTCCATCTATCC			
panN gene encoding for isoepoxydon dehydrogenase (IDH) enzyme	patN F	CAATGTGTCGTACTGTGCCC	52°C	600	Paterson et al. (2003)
	patN R	ACCTTCAGTCGCTGTTCCTC			

Plates were placed in the migration tank, loaded with 15 ml migration mixture, containing toluene: ethyl acetate: formic acid, 60:30:10 (v/v/v) (Nayaka et al., 2013). The standard ochratoxin A solution was used as control. After migration, the plates were exposed to 365 nm UV light and analyzed according to Vujanovic & Ben Mansour (2011) protocol. To determine the presence of mycotoxigenic compounds in samples, the retention factor (Rf) was calculated for standard mycotoxins. This important parameter is calculated after chromatographic separation, and indicates the relative, specific displacement of each compound in the sample, along the solvent migration front. The Rf values are in between 0 and 1, while each TLC migrated compound has a different, specific value. However, the Rf can depend on the TLC parameters, such as temperature, layer thickness, degree of solvent saturation in the separation chamber, the volume of the sample, as well as the type of mobile and stationary phase. The formula for calculating the Rf value is given by the ratio of the compound migration compare to the migration front on the chromatographic plate, as follows:

$$Rf = \frac{\text{Distance traveled by the compound (cm)}}{\text{Distance traveled by the migration front (cm)}}$$

***In vitro* antagonistic assay**

Dual culture technique was performed to evaluate potential probiotic bacteria against blue mold development.

The test was performed *in vitro*, on PDA medium.

Eight bacterial strains were tested: *Bacillus* sp. B4, *B. subtilis* B5, B6, and *B. amyloliquefaciens* BW, BIR, BPA, OS15, and OS17.

The bacterial strains were inoculated as spots, four per plate, equidistant one from each other, and 2 cm away from the center of the plate. Then, the blue mold was inoculated in the center of the Petri dish.

Penicillium inoculum was prepared as conidial suspension inv sterile distilled water, supplemented with three drops of Tween 80.

The suspension was prepared as 10^7 cfu/ml, and 25 µl conidial suspension was used to inoculate each plate.

Fungal control was also prepared, were the blue mold was grown on PDA, and incubated in similar conditions.

The antifungal activity was evaluated after ten days of incubation at room temperature, by measuring the fungal growth in both control and test plates and calculating fungal inhibitory efficacy, according to Boiu-Sicuia et al. (2021).

RESULTS AND DISCUSSIONS

Fungal contaminants quantification

Fungal load in the Sudanese feed samples was quantified as 10^3 cfu/g of sample. Only molds were counted, while yeasts were not taken into consideration (Figure 1).

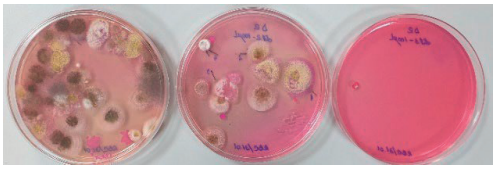

Figure 1. Mold colonies grown from different dilution of D2 Sudanese feed sample on RBC media

Blue molds enumeration

Each colony with typical color and morphology isolated from one of the poultry feed samples was considered an isolate.

At the end of the incubation period, which lasted 4 days at 28°C, the blue-like molds were purified. Based on the macroscopic features and colony morphology, only four strains (A3-2, A3-4, C1-1, and C3-1) were taken as potential blue molds (Figure 2).

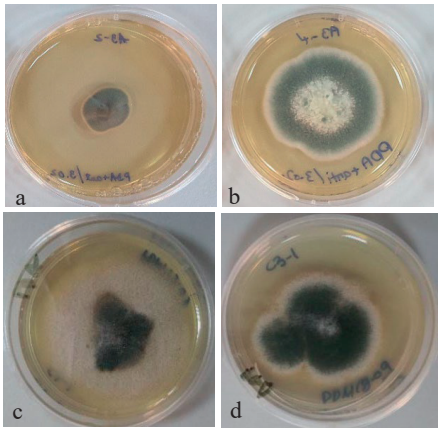

Figure 2. Blue molds contaminants isolated from Sudanese feed sources on PDA:
a. A3-2; b. A3-4; c. C1-1; d. C3-1 strains

Under microscopic evaluations, only A3-4 strain revealed *Penicillium* sp. characteristics, showing brush shape conidiophores (Figure 3a). Two other strains (C1-1 and C3-1) revealed vesiculated conidiophores (Figure 3b), while the A3-2 strain developed branched, brown pigmented, conidiophores (Figure 3c).

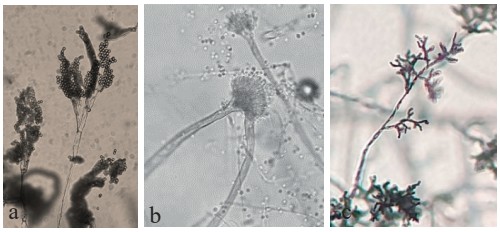

Figure 3. Conidiophoses and conidia:
a. *Penicillium* sp.; b. *Aspergillus* sp.; c. *Cladosporium* sp.

In other African countries, different multi-mycotoxin producing fungi were found as post-harvest contaminants of sorghum grains. *Aspergillus* and *Penicillium* are among the fungal contaminants recovered from infected grains (Mohammed et al., 2022; Deligeorgakis et al., 2023).

Generally, the *Cladosporium* species are not included among the major toxigenic fungi.

However, as contaminant is mentioned to be present of the sorghum grains produced in Africa (Pambuka et al., 2021).

Molecular identification

Based on the sequence analysis of the ITS1-5,8S-ITS2 region the mold strains A3-2, A3-4, C1-1, and C3-1 were attributed to 3 species (Table 2).

Fungal identification based on molecular means confirmed the microbiologic results, highlighting the presence of fungal species with mycotoxigenic potential.

Table 2. Fungal strains identification

Fungal strain	Molecular identification	Sequence length	Reference strain/ NCBI accession no.	Query Cover	Identity Percent
A3-2	*Cladosporium tenuissimum*	524 bp	BFMY-2 / MT573533.1	100%	100.00%
A3-4	*Penicillium polonicum*	503 bp	DUCC5746 / MT582786.1	100%	99.60%
C1-1	*Aspergillus nidulans*	523 bp	CBS 114 63 / MH858232.1	99%	99.81%
C3-1	*Aspergillus nidulans*	530 bp	CBS 129375 / MH865286.1	100%	100.00%

Molecular identification of mycotoxin encoding genes

Several genes encoding for mycotoxin synthesis were screened by classical PCR technique. The four tested fungal strains revealing the lack of *nor-1* (*aflD*), *ver-1* (*aflM*), *omt-A* (*aflP*), and *aflR* genes. As these genes are encoding for key enzymes involved in aflatoxin biosynthetic pathway, the results suggest that the studied strains are not aflatoxin producers. These findings are highly encouraging, considering that *A. nidulans* could produce the carcinogenic sterigmatocystin mycotoxin (encoded by *omt-A* gene), which is a precursor of the highly dangerous aflatoxins (Keller et al., 1994).

The other two studied genes, *ota.nps*, encoding for ochratoxin A, and *patN*, encoding for patulin were both found to be present in *P. polonicum* A3-4 strain (Figure 4 a, b).

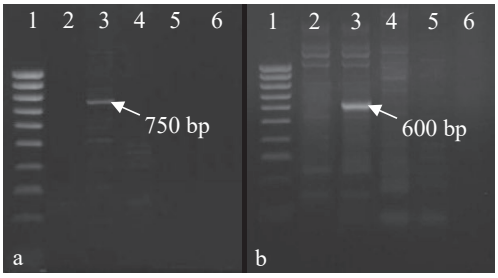

Figure 4. Highlighting the presence of the *ota.nps* (a) and *patN* (b) genes in *P. polonicum* A3-4 Sudanese strain
Legend: Line 1 = Molecular marker of 100bp; Line 2 = A3-2 strain; Line 3 = A3-4 strain; Line 4 = C1-1 strain; Line 5 = C3-1 strain; and Line 6 = Negative Control

Considering that *P. polonicum* is used as biosynthetic source of ochratoxin A (Mantle et al., 2016), the presence of *ota.nps* gene is not surprising. Beside ochratoxin A, *P. polonicum* fungi could produce various other mycotoxigenic metabolites, such as anacine, patulin, penicillic acid, verrucosidin, and 3-methoxyviridicatin (Núñez et al., 2000; Ding et al., 2013). Therefore, finding *patN* gene that encodes for patulin synthesis is considered to be a regular characteristic.

Mycotoxin analysis

Performing the TLC methodology for ochratoxin A detection, in the fourth studied fungal strains, confirmed the results of the molecular analysis. Ochratoxin A being found present only in the crude extract of *P. polonicum* A3-4 strain (Figure 5). Nether of the other strains of *C. tenuissimum* or *A. nidulans* revealing the presence on this toxin in their crude extracts.

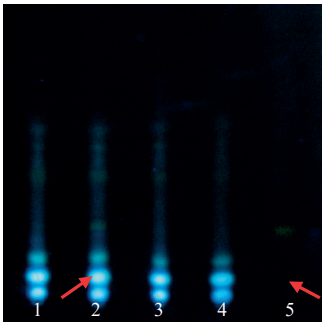

Figure 5. TLC chromatogram exposed to UV light revealing ochratoxin A compound (red arrows)
Legend: Line 1 = A3-2 strain; Line 2 = A3-4 strain; Line 3 = C1-1 strain; Line 4 = C3-1 strain, and Line 5 = ochratoxin A standard

Based on the calculated Rf value for the mycotoxin standard used, the green spot of 0.27 Rf is confirming the presence of ochratoxin A, only in the crude extract of A3-4 strain.

***Penicillium* biocontrol solution**

Bacterial biocontrol potential against mycotoxigenic *P. polonicum* A3-4 isolate was evaluated *in vitro* conditions.

Most of the tested strains, previously characterized as biocontrol strains (Sicuia, 2013) revealed good antifungal activity against *P. polonicum* A3-4, showing 78.09 to 82.02% mycelia inhibitory efficacy (Table 3).

Table 3. *P. polonicum* mycelia growth inhibition

Bacterial strain	Clear inhibition zone	Bacterial efficacy in fungal growth inhibition
B4	1.5 cm	54.67%
B5	2.0 cm	78.16%
B6	1.0 cm	69.34%
BW	0.5 cm	78.09%
BIR	1.5 cm	82.04%
BPA	3.5 cm	79.41%
OS15	2.5 cm	79.41%
OS17	1.5 cm	79.41%

Although some of the bacterial tested strains, such as B4 and B6, were promising biocontrol agents against other toxigenic fungi (Grosu et al., 2015), they revealed lower antifungal activity on *P. polonicum* A3-4 compared to the other tested strains.

CONCLUSIONS

The prevalence of fungal contaminants in Sudanese poultry feed revealed to be 10^3 cfu/g of sample. Among the fungal contaminants only 4 isolates revealed blue-like appearance. One of these isolates (A3-4) for identified as *P. polonicum* and found to be ochratoxin A and patulin producer. To reduce the growth of this pathogen, several *B. amyloliquefaciens* strains were found efficient inhibitor. Best results (82.04% efficacy) were obtained with the BIR biocontrol strain.

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