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Research Paper

Molecular Identification of Fungi and Determination of Aflatoxin B1 in Dried Tomatoes

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Abstract— Occurrence of fungi and their secondary metabolites such as mycotoxins in tomatoes is of great public health concern. World economies as well as human and animal health are significantly affected by their presence. It is therefore imperative to understand the species of fungi and the types of mycotoxins produced by them. This study is aimed at isolating, characterizing, and comparing the fungal load as well as quantifying aflatoxin B1 levels produced in dried tomatoes sold within Gusau metropolis. A total of 23 tomato samples were randomly obtained from different markets in the study area. Of the samples, 39 fungal isolates were found; three of the fungal isolates belong to the genus *Aspergillus. Aspergillus flavus*, which is known largely for the production of aflatoxins, was identified as the predominant occurring fungus with 48.7% (n = 19), and the least occurring fungi were *Curvularia americana* and *Fomitoptis maliae* with 2.6% (n = 1) each. The rRNA gene of the isolates was amplified by PCR and sequenced. The sequence data were deposited in the NCBI database, and accession numbers were assigned to each isolate. Aflatoxin B1 (AFB1) was detected and quantified from the samples, and findings from this study indicated that AFB1 was present in all the samples analyzed. Also, 43.48% of the samples exceeded the maximum permissible number of 20 µg/kg set by the Standard Organization of Nigeria. In general, this result suggests that the presence of AFB1 in all the samples confirmed that *Aspergillus flavus* is the highest aflatoxins-producing fungus. It is therefore recommended that food products be screened for the types of fungal species and the presence of mycotoxins for food and public health safety.

Keywords— Aflatoxins, fungi, tomato, ELISA, Mycotoxins

1. Introduction

Contamination of agricultural products by mycotoxins is a serious global health problem. More worrisome is the high stability of the mycotoxins, which enables them to contaminate agricultural products from cropping to storage of the harvested products [1]. Mycotoxins have been described as hazardous secondary metabolites of mostly filamentous fungi [2]. Their adverse effects do not cause damage to the agricultural crops alone but also affect human and animal health, which in turn could lead to economic loss or death [3], and their occurrence in raw crops remains relatively higher than in finished products [4]. Currently, there are an estimated number of 300 mycotoxins which have been identified. However, only six are frequently detected in consumables and other agricultural products [2]. Aflatoxin is the most significant class of mycotoxins in agriculture because of its recurrent occurrence in food and other products. With more than 20 discovered aflatoxins, only aflatoxin B1, B2, G1, and G2 are the most significant [5]. Aflatoxins are toxic, carcinogenic, and mutagenic compounds produced by fungal species of the genus *Aspergillus* [6]. In the contamination of agricultural products, *A. flavus* and *A. parasiticus* are reportedly implicated [7].

Tomato has been described as one of the major crops consumed globally. It provides nutrients required by humans, such as vitamins, minerals, antioxidants, and carbohydrates [8]–[9]. A trend analysis of tomato production in Nigeria conducted by Ugonna *et al.,* [10] reported that 50% of tomatoes cultivated do not reach their desired destination; this is due to many factors, which include attack by pathogens such as AFB1-producing fungi. Ghosh [11] discovered that most tomato samples accessed for microbial contamination, harboured fungi more than bacteria, and contamination and spoilage of tomatoes by pathogens result in economic loss and food poisoning [12].

Fungi are a group of diverse microorganisms that inhabit different plant parts (leaves, roots, stems, and seeds) and other environmental sources like soil, water, and food [13]. They play a crucial role in ecosystem processes, but their diversity and biogeographic pattern are poorly understood [14]. Similarly, Rodriguez *et al.,* [15] asserted that there is a dearth of information on the diversity and ecological

significance of fungi that has been poorly characterized despite over 100 years of published research within the realm of scientific literature.

The conduct of this study was compelled by the high demands for tomato in meeting nutritional requirements, the occurrences of fungal contaminants and their secondary metabolites, and the presence of heavy metals in food, as well as the paucity of information regarding the level of aflatoxins produced in dried tomato within the study area [16]–[18]. The study therefore seeks to isolate and explore fungal diversity in dried tomatoes and to also determine the level of aflatoxin B1 produced in the dried tomatoes sold within Gusau metropolis. The findings of this study will provide baseline information on the level of aflatoxin B1 and fungal contaminants in dried tomatoes, which will ultimately guide food safety regulatory agencies in Nigeria in enforcing standards for consumables, especially tomatoes.

2. Experimental Method/Procedure/Design

Twenty-three (23) samples of dried, ready-to-use tomatoes were randomly obtained from various markets within Gusau metropolis. The samples were collected in sterile plastic bags and stored at -4 °C in the refrigerator until further analysis.

2.1 Isolation and Identification of Fungi from Tomato Samples

Fungi associated with the dried tomato samples were isolated and identified according to the procedure of Odelade and Oladeji [19], with slight modifications. Briefly, each tomato sample was aseptically cut using sterile blade and 10 g each was placed into Erlenmeyer flask containing 90 ml of sterile distilled water. The flasks were maintained at 120 rpm for 30 minutes on an orbital shaker to allow for thorough mixing. Thereafter, 1 ml of the sample was mixed with 9ml of of sterile distilled water and serially diluted. Pour plate method was adopted for the isolation of fungi on potato dextrose agar (PDA) plates incubated at 25 °C for five days. Fungal isolates were observed and subcultured repeatedly until pure cultures were obtained. The characterization of the fungal isolates was based on colony and cell morphology using wet mount preparation according to the mycological atlas of Sarah *et al.*, [20]. The pure cultures of each of the fungal isolates were stored on a PDA slant at -4 °C in the refrigerator for further analysis.

2.2 Molecular Characterization of the Fungal Isolates

To obtain a high yield of the nucleic acid of the isolates, each isolate was subcultured in potato dextrose broth [21]. DNA extraction was carried out using a fungi/yeast RNA/DNA purification kit (NORGEN BIOTEK CORP., Canada) according to the manufacturer's instructions. The amplification of the internal transcribed space (ITS) region of the rRNA gene of all the fungal isolates was performed using primers ITS1: 5' (TCC GTA GGT GAA CCT GCG G) 3' and ITS4: 5' (TCC TCC GCT TAT TGA TAT GC) 3'. The PCR condition was: predenaturation at 95 ^{0}C for 5 minutes, then denaturation at 96 ^{0}C in 30 cycles for 30 seconds; this is followed by annealing at 62 ^{0}C for 30 seconds; extension for 30 seconds at 72 0C; and a final extension at 72 ^{0}C for 10 minutes. 1% agarose gel electrophoresis was used to detect the PCR products, and the products were sequenced using the services of Inqaba Biotec West Africa LTD, Ibadan, Oyo State, Nigeria. The results were used to determine the maximum score, total score, query cover, and percentage identity of the sequences amplified using the Basic Local Alignment Search Tool (BLAST) of the NCBI website (https://www.ncbi.nlm.nih.gov/), and the sequences were deposited in the NCBI database and accession numbers were assigned accordingly [22].

2.3 Sample Preparation for the Detection of Aflatoxin B1

The method described by [23] was used for aflatoxin extraction with slight modifications. Briefly, 50 g of each of the sample was grinded into a fine powder. 100 ml of 70% methanol was mixed with 20g of the grinded sample into a 250 ml capacity clean conical flask. The mixture was stirred for 30 minutes at a speed of 150 rpm. Whatman filter paper was used to filter the sample, and aflatoxin B1 extract was obtained from the filtrate.

2.4 Aflatoxin B1 Assay and Quantification

Aflatoxin B1 ELISA kit obtained from ICRISAT was used for the assay, and quantification was performed as described by Abdullahi *et al.,* [24].

3. Results

Table 1.0 shows the occurrence of fungal isolates from dried tomato samples. Thirty nine (39) fungal isolates were recovered from the 23 dried tomato samples analyzed. Identification of the fungal isolates was based on the colony morphological and cellular characteristics. *Aspergillus flavus* showed the highest occurrence of 19 (48.7%), the least occurring fungi being *Curvularia americana* and *Fomitopsis meliae,* with 1 (2.6%) occurrence each.

Table1.0: occurrence of fungi in dried tomato samples

S/N	Fungal Species	No. of Isolates	Frequency (%)
1.	*Curvularia americana*	1	2.6
2.	*Aspergillus flavus*	19	48.7
3.	*Fomitopsis meliae*	1	2.6
4.	*Chaetomium globosum*	3	7.7
5.	*Aspergillus nidulans*	8	20.5
6.	*Aspergillus sydowii*	7	17.9
	TOTAL	**39**	**100**

Table 2 shows the NCBI accession numbers assigned to each fungal isolate. PCR was used for the amplification of rRNA gene of the ITS region of each of the isolate, and sequencing was further performed to obtain the DNA sequences for each fungal isolate. These were used to determine the percentage identity, query cover, and matched organisms.

Table 2: NCBI accession numbers and matched organisms

NCBI No.	ID (%)	QC (%)	Organism
OQ866339	99.81	99	*C. americana*
OQ866340	99.64	98	*A. flavus*
OQ866341	98.40	99	*F. meliae*
OQ866342	98.81	97	*C. globosum*
OQ866343	100	99	*A. nidulans*
6. OQ8663	99.62	99	*A. sydowii*

Key: ID= Percentage Identity, QC= Query Cover

Table 3 shows the levels of aflatoxin B1 in the 23 dried tomato samples analyzed at various concentrations. The mean aflatoxin B1 concentration was 23.2 µg/kg. From the results obtained, sample S22 showed the highest aflatoxins B1 concentration of 50.2 µg/kg, while samples S18 and S21 had the least concentration of AFB1 of 8.5 µg/kg each. The result also shows that 43.48% of the samples exceeded the maximum permissible limit of 20 µg/kg as set out by the Standard Organization of Nigeria (SON).

Table 3: comparison of the concentrations of aflatoxin b1 in dried tomato samples with the son standard (µg/kg)

Sample	AfB1 Conc. (µg/kg)	SON Compliance
S1	26.5	ESL
S2	12.1	WSL
S3	15.0	WSL
S4	20.0	WSL
S5	40.6	ESL
S6	17.3	WSL
S7	12.1	WSL
S8	24.7	ESL
S9	37.8	ESL
S10	32.8	ESL
S11	35.2	ESL
S12	11.3	WSL
S13	17.3	WSL
S14	40.6	ESL
S15	13.0	WSL
S16	17.3	WSL
S17	32.8	ESL
S18	8.5	WSL
S19	30.6	ESL
S20	15.0	WSL
S21	8.5	WSL
S22	50.2	ESL
S23	15.0	WSL

Key: SON: Standard Organization of Nigeria, ESL: Exceeds SON Limit, WSL: Within SON Limit, SON maximum permissible limit of aflatoxins = 20 µg/kg

4. Discussion

Tomatoes are naturally susceptible to fungal spoilage because of their high nutrient and water content. This enhances water activity and provides nutrients for fungal growth and proliferation. With increased fungal growth, the production and secretion of secondary metabolites (mycotoxins) are further increased [12]. In this study, fungal contamination of dried tomatoes revealed the presence of *Aspergillus flavus* (48.7%), *Aspergillus nidulans* (20.5%), *Aspergillus sydowii* (17.9%), *Chaetomium globosum* (7.7%), *Curvularia americana* (2.6%), and *Fomitopsis meliae* (2.6%). The diversity of these fungi is of great public health concern since *Aspergillus flavus* is known for the production of aflatoxins [25]. In addition to the occurrence of *A. flavus* in dried tomatoes, it has also been reported in other fruits such as cucumber, watermelon, and chili [26], [19], and [27]. The highest occurrence of *Aspergillus flavus* in this study corroborates the findings of Akomolafe *et al.,* [28], who reported *Aspergillus flavu*s as the most predominant fungal isolate in dried tomatoes in southern Nigeria. Also, a previous study conducted by Shinkafi *et al.,* [29] on the diversity of fungal isolates in dried tomatoes revealed the presence of all the fungal isolates in this study and some others not identified in this work. Penton *et al.,* [30] recounted that the diversity of both the bacterial and fungal communities was significantly affected by sample size; therefore, the differences in the diversity of fungi between the current and previous studies could be a result of the limitation of the sample size in the current work.

Complete or partial variation in the diversity of fungi in tomatoes or other fruits could be due to differences in climatic conditions and geographical locations [31]–[33]. For instance, Mailafia *et al.,* [34] reported *Aspergillus flavus* as the least occurring fungus in spoilt tomatoes, having only 5%. Similarly, the work of Onuorah and Orji [12] reported *Aspergillus niger* as the most commonly occurring fungus associated with spoilage of post-harvest tomatoes. Additionally, Oshita *et al.,* [35] reported fungal diversity in tomatoes, which differs completely from the present study in Japan.

According to the results of this study, there are varying levels of Aflatoxin B1 contamination in all the dried tomato samples analyzed. This is consistent with the previous study conducted by Safavizadeh *et al.,* [36] on food samples having AFB1 contamination in all the samples, including tomatoes. However, the result disagrees with that of Suleiman *et al.,* [37], who reported no AFB1 contamination in all 25 samples of dried tomato analyzed in Minna, Niger State. This could be attributed to the storage of the aflatoxins extracts at -200 °C, which could affect the stability of the aflatoxins and render them undetectable in the samples.

The findings of this work also revealed that 43.48% of the samples analyzed exceeded the maximum allowable limit of the Standard Organization of Nigeria (20 µg/kg). This level could be within the limits of aflatoxins acceptable or otherwise in some countries because different counties and regions have standards for the maximum allowable limit of aflatoxins. The limit is usually set based on risk assessment and scientific evidence of the levels that are considered safe for products to be consumed [38]. Additionally, variation in climate and other environmental conditions such as temperature, water, and light may influence the lifecycle of fungi and aflatoxins production as well as the maximum allowable limits [39]–[41].

The high levels of aflatoxin B1 in all the samples in the present study may be attributed to a number of factors, such as a possible source of contamination in the drying process, which can give a favourable growth condition to the aflatoxin-producing fungi. According to Shinkafi *et al.,* [29], the most popular method of drying tomatoes in northern Nigeria is sun-drying, which is mostly done in an open air space on a mat; sometimes, this is done on a bare floor or any surface whose hygiene is not certain. Exposing tomatoes to the air may cause fungal contamination because of the complex mixture of microorganisms contained in the air [42]. This therefore confirms the contamination of aflatoxins B1 in all the samples in this study, and it implies tendencies for health risks for its consumers.

5. Conclusion and Future Scope

A high level of contamination with aflatoxins B1 was observed in all the dried tomato samples analyzed, which confirms the presence of *Aspergillus flavus* as the predominant fungal contaminant in the samples. The tomato producers are recommended to adopt new strategies in the storage of tomatoes, thereby avoiding sun-drying on bare floors or fully exposed. Further research direction is also recommended to prevent aflatoxins biosynthesis during the storage and drying of tomatoes.

Conflict of Interest
All authors declare no conflict of interest.

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Authors' Contributions
Salisu, N., conceived the idea and researched literature, Salisu, N., Ukwaja, V. C., and Sakariyau, W. A., involved in the protocol development, result analysis, and draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

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- must be **original**
- must be **previously unpublished research results**
- must be **experimental or theoretical**
- must be in **the journal's prescribed Word template**
- and will be **peer-reviewed**
- may not be **considered for publication elsewhere at any time during the review period**