# Fungal molecular identification and total aflatoxin assessment in stored peanut seeds in Kwara State, Nigeria

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## Abstract

One kilogram each of peanut (*Arachis hypogaea* L.) seeds were collected from each of six popular markets with high patronage in Kwara State, North-Central Nigeria, namely Alapa, Ganmo, Ipata, Kaiama, Share and Tsaragi markets. There is lack of reliable information on the fungal species and level of total aflatoxins (TAF) in the peanut seeds sold in these markets. The fungal species associated with the samples were isolated using serial dilution and pour plating methods, and then identified morphologically and molecularly, after which the TAF in each sample was evaluated using the direct competitive enzyme-linked immunosorbent assay method (ELISA). A total of five fungal species *Aspergillus flavus, A. oryzae, Mucor indicus, Rhizopus oryzae* and *R. arrhizus* were isolated from the collected samples. Aflatoxins were present in 66% of the examined peanut seeds at varying values. The highest TAF content *viz.* 2.5 part per billion (ppb) was recorded in the peanut seeds from Share market, while the lowest TAF content (0.9 ppb) was recorded in the seeds from Alapa market. The TAF content values obtained in this study were within the permissible limits (20 ppb) approved by the National Agency for Food and Drug Administration and Control (NAFDAC), as well as the 10 ppb approved by the Codex Alimentarius Commission (CAC) for Peanut seeds in Nigeria. However, prolonged and consistent consumption of peanut seeds with these doses of TAF may result in accumulation of aflatoxins in the body, thereby posing a potential health challenge over time.

**Keywords:** Fungal species, Kwara State, Nigeria, Peanut seeds, Total aflatoxin.

## Introduction

Peanut, a significant economic crop cultivated in Nigeria, is a pulse plant thriving in subtropical and semi-arid regions globally. Its origin can be traced back to Southern America, but presently, China, India, Nigeria, and the United States are the leading global producers, contributing more than 95% of the overall world production in 2020 (Ahmed *et al*., 2020). Within Africa, Nigeria holds the foremost position as a peanut producer, contributing approximately 10% to the global production and accounting for 39% of Africa's total output in 2020 (Jekayinfa *et al*., 2020).

Peanut seeds are rich in monounsaturated fats, thereby promoting heart health by lowering bad cholesterol, and act as antioxidant boost, blood sugar control, weight management, nutrient powerhouse amongst others; its primary utilization is in the production of vegetable oil which is later processed into items such as peanut butter, confectionery, snack foods, meat extenders, soups, and desserts (Madilo *et al*., 2023). In Nigeria, there are documented instances of more than ten processed peanut products, encompassing native meat condiments, peanut pastes, cakes, and cooking oil, serving various purposes (Abdulrahaman *et al*., 2014; Tyohemba *et al*., 2021). Nigerians eat a lot of peanuts with an average daily consumption of 5–10 g per person in North-Central Nigeria (Mekonnen *et al*., 2021).

Certain toxic byproducts such as aflatoxins are produced by certain fungi such as *Aspergillus flavus* and *A. parasiticus*, which flourish in environments containing grains, hay, soil and decaying vegetation (Asghar *et al*., 2020). These byproducts are commonly detected in inadequately stored essential food items (Kumar *et al*., 2021). Peanut can be specifically exposed to aflatoxin contamination due to various factors such as field contamination, post-harvest drying and storage, as well as transportation, while aflatoxin levels are influenced by farming methods, physical injury, entomofauna and avian infestations, weather conditions and soil properties (Bhatnagar-panwar *et al*., 2020). When animals consume food contaminated with aflatoxins, these can be transferred to humans through meat, dairy products and eggs (Sipos *et al*., 2021; Popescu *et al*., 2022). Aflatoxin, particularly aflatoxin B1, is a powerful carcinogen in animals, among other consequences (Alameri *et al*., 2023). It has also been observed that aflatoxins weaken both animals as well as human immunity. Aflatoxin exposure significantly increases stunting and underweight in children, particularly in those under age three. Additionally, the slower pace of recovery following a protein deficiency (kwashiorkor) has been linked to aflatoxins (Popescu *et al*., 2022). Aflatoxins generally impair animal

growth by interfering with protein synthesis and have been linked to a number of human nutritional-related disorders (Awuchi *et al*., 2022). Aflatoxicosis thus poses a serious health concern to consumers (Jallow *et al*., 2021).

In Nigeria, the National Agency for Food and Drug Administration and Control (NAFDAC) approved allowable threshold for total aflatoxins in any food item intended for human consumption is 20 ppb, while the Codex Alimentarius Commission, CAC approved the maximum limit for TAF is 10 ppb (Ubwa, 2014; Mothiba *et al*., 2023). Reliable information on the TAF levels in peanut seeds sold in various markets in Kwara State, from which the study samples were collected, is lacking. Therefore, this study was carried out to bridge this gap.

## Materials and Methods

**Collection of stored peanut seeds**

Stored peanut seeds (1 kg each) were purchased from Alapa, Ganmo, Ipata, Kaiama, Share and Tsaragi markets, all located in Kwara State. They were stored separately in labeled sterile paper bags and taken to the laboratory of the Department of Plant Biology, University of Ilorin, Ilorin, Nigeria, where each sample was divided equally into batch A (for fungal isolation) and batch B (for total aflatoxin analysis). Batch A was stored at 4 °C, while batch B was stored at −20 °C in the laboratory (Esan *et al.*, 2020).

**Culture media preparation, fungal isolation and morphological identification**

Following the manufacturer's specifications, 39 g of potato dextrose agar (PDA) powder was dissolved by mixing it with 1 L of distilled water in a conical flask. After it was well mixed, autoclaved at 121 °C for 15 min, allowed to cool down to 45 °C, 20 mL was aseptically poured onto sterile Petri plates. Prior to pouring the molten PDA into the culture plate, 2 mL of streptomycin was introduced into 250 mL of molten PDA to inhibit bacterial growth. The isolation process employed the dilution plate technique, as detailed in the study by Akharenegbe *et al.* (2022). In a test tube, 9 mL of sterile water was mixed with one gram of ground peanut seeds, followed by a process of decimal dilution for the solution.

The count of fungal colonies on the plates was tallied, and the determination of colony-forming units per gram (cfu $g^{-1}$) in the analyzed samples followed the procedure outlined by Abdulla (2013). The fungal colonies on each plate were sub-cultured on fresh PDA to obtain pure cultures of fungi. Percentage of occurrence of each of the pure fungal isolate was calculated as described by Adebola *et al*. (2014). Following that, isolates in their pure form were identified morphologically using the methods reported by Fawole and Oso (2007) and Kidd *et al*. (2023). To confirm the morphology-based designations, representative isolates were selected, and molecular analysis was performed.

**Molecular identification**

Zymo Research DNA kit was used for the total genomic DNA extraction of each of the representative isolates according to a step-wise protocol described by the manufacturer. According to Liu *et al*. (2007), amplification of the Internal Transcribed Spacer (ITS) region of each was done using unique primer combination: ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC -3′) at Inqaba Biotec, Ibadan, Nigeria**.** A thermal cycler PCR equipment was used for this, and subsequent sequencing of the amplification products was done at the same laboratory. Using the methods outlined by Stucky (2012), a consensus DNA sequence was generated from the forward as well as reverse sequences using the Seqtrace program (Seqtrace-win-0.9.0). Nucleotide sequence (ITS) of each fungal isolate was matched with the National Centre for Biotechnology Information's (NCBI) GenBank database employing the Basic Local Alignments Search Tool for Nucleotides (BLASTN) Sequences, which can be accessed on the GenBank repository web page (https://www.ncbi.nlm.nih.gov/). Phylogenetic trees were then constructed by comparing the query sequences with subject sequences obtained from NCBI, employing Molecular Evolutionary Genetics Analysis (MEGA version 11.0) and using the maximum likelihood method.

**Preparation of peanut seeds for aflatoxin extraction**

The protocol outlined by Amai *et al*. (2021) was applied at the NAFDAC Laboratory, Kaduna, Nigeria. Twenty peanut seeds were finely ground, and 2 g of the ground material was weighed into 10 mL of 70% methanol for the extraction process. Utilizing a shaking device, the mixtures were agitated for 10 min at room temperature before being allowed to get settled. The homogenate was then passed through Whatman No. 1 filter paper for filtering. Distilled water was added to the resulting filtrate to dilute it.

**Detection and quantification of aflatoxin**

Total aflatoxin ELISA kit was obtained from R-Biopharm AG, and aflatoxins were detected and quantified in accordance with Ekpakpale *et al*. (2021).

## Results

**Fungal counts and morphological identification of the isolates**

The fungal counts obtained in this study

ranged from $2.3 \times 10^5$ cfu $g^{-1}$ for peanut seeds from Ipata to $58.33 \times 10^5$ cfu $g^{-1}$ for peanut seeds from Share market (Table 1). Five fungal species, spanning three genera, were identified from the peanut seeds examined in this study. Based on their morphological features, they were tentatively identified as *Aspergillus flavus*, *A. oryzae*, *Mucor* sp., *Rhizopus oryzae* and *R. arrhizus.*

**Percentages of occurrence of the fungal isolates**

The percentage occurrence of *A. flavus* was the highest (56%), while the value of the same parameter for the other isolates were *A. oryzae* (26%), *Mucor* sp. (11%), *Rhizopus arrhizus* (4%), and *R. oryzae* (3%) (Table 2).

**Molecular identification of the fungal isolates**

Table 3 displays the unique NCBI accession numbers assigned to individual fungal isolates. PCR was utilized to amplify the rRNA gene within the ITS region of each isolate, followed by sequencing to acquire the DNA sequences. These sequences were then employed to ascertain the percentage identity, query cover, and matched organisms. Figures 1–5 illustrate the phylogenetic relationships of the query sequences from the five isolated fungal species in comparison to various subject sequences found in the NCBI database, thereby providing a comprehensive view of their genetic similarities and differences.

**Total aflatoxin**

The total aflatoxin levels (ppb) in the peanut seeds analyzed in this research are presented in Table 4**.** Out of the 20 peanut seeds from Share market, 17 had aflatoxins, the highest number, with mean aflatoxin concentration of 2.5 ppb or 29% (the highest), while out of the 20 peanut seeds from Alapa market, only 11 had aflatoxin, the lowest number with mean aflatoxin concentration of 0.9 ppb or 11% (the lowest) (Table 4). The total number of peanut seeds containing aflatoxins out of the 120 analyzed was 79, with total aflatoxins concentration of 8.5 ppb (Table 4).

## Discussion

The mycoflora and aflatoxins concentrations in stored peanut seeds obtained from 6 popular markets in Kwara State were evaluated in this study. Results obtained showed the presence of fungal species in all peanut seeds screened with those from Share market having the highest fungal count ($58.33 \times 10^5$ cfu $g^{-1}$), and the peanut seeds from Ipata market having the lowest fungal count ($2.33 \times 10^5$ cfu $g^{-1}$). The disparity in the fungal count as recorded in this study may be due to difference in the storage facilities available in these markets. The results are in agreement with that of Adetunji *et al*. (2018) which reported fungal counts ranging from $4.00 \times 10^3$ cfu $g^{-1}$ for Fidiwo market to $24 \times 10^3$ cfu $g^{-1}$ for Mowe market in Ogun state.

The isolated fungal species associated with the screened peanut seeds were identified molecularly as *Aspergillus flavus*, *A. oryzae*, *Mucor indicus*, *Rhizopus oryzae* and *R. arrhizus*. None of the individual query cover and percentage identity was less than 98% and 97%, respectively in this study, thereby authenticating their identities. According to Raja *et al*. (2017), dependable identification through molecular techniques involves achieving a query cover of at least 80% and a percentage identity of at least 97% in the comparison of isolated fungal species sequences with those in the NCBI database, utilizing the Basic Local Alignment Search Tool for Nucleotides (BLASTN) Sequences. Use of molecular markers such as ITS and others is very popular nowadays for correct identification of fungal species (Khan *et al.*, 2021; Khan and Javaid, 2022).

The list of fungi isolated in this study agrees with those identified in the report of Kigigha *et al*. (2016) discovered *Aspergillus* spp., *Fusarium* spp., *Mucor* spp., and *Rhizopus* spp. Abuga (2014) identified several fungal genera, such as *Aspergillus*, *Fusarium*, *Mucor*, *Penicillium*, and *Rhizopus*, in peanut seeds obtained from the capital market in Aliero, Kebbi State. Conversely, Ezekiel *et al*. (2018) noted the presence of *A. flavus*, *A. tamarii*, and *A. parasiticus* in roasted peanuts in southwestern Nigeria. In various other studies, species of *Aspergillus*, *Penicillium* and *Mucor* were reported from stored seeds of other economically important crops such as wheat, rice (Shafique *et al.*, 2007; Butt *et al.*, 2011; Khan and Javaid, 2022).

In this study, *Aspergillus flavus* was most common (56%), while *Rhizopus oryzae* was the least (3%) in the peanut seeds screened in this study. The dominance of *Aspergillus flavus* in this study agrees with report of the Sangoyomi (2016) which indicated *Aspergillus* flavus as the predominant fungal species on peanut seeds obtained in Oyo and Osun States. The primary fungi affecting the nutritional value of Peanut seeds which are marketed in the cities of Benin and Yenagoa, accordingly, are *Aspergillus* spp. (Akinnibosun and Osawaru, 2015; Kigigha *et al*., 2016), similar to the current findings.

The results from this study indicate that 66% of the examined peanut seeds tested positive for aflatoxins at concentrations ranging from 0.9 to 2.5 ppb. This differs from the observations of Bakhiet and Musa (2011), who noted that 58% of peanut-based products in Sudan showed aflatoxin positivity within a range of 17.57 to 404.00 ppb. Similarly, Uzeh and Adebowale (2021) documented a total aflatoxin concentration in Peanut and locally processed peanut butter samples in Lagos State ranging from 373.6 to 6741.6 ppb. Several studies have reported relatively lower total aflatoxin concentrations. Mupunga *et al.* (2014) examined peanut-based products in Zimbabwe and recorded

levels ranging from 6.1 to 247 ng $g^{-1}$. Similarly, Abdulhameed (2014) reported total aflatoxin concentrations in peanut seeds from the eastern Mediterranean region of Turkey as ranging from 0 to 0.192 ppb.

## Conclusion

The research findings revealed that peanut seeds in the investigated markets were contaminated with both mycoflora and TAF, with their concentrations however remaining within the thresholds established by both local and global regulatory bodies. Despite these, there is the need for proper storage of peanut seeds.

## Contribution of authors

IA carried out the experiments while GSO supervised the research work. Both authors prepared the manuscript.

## Conflict of interest

Authors declared that there is no conflict of interest.

**Table 1:** Number of colonies forming units on peanut seeds.

| Markets | Fungal count (× $10^5$ cfu $g^{-1}$) |
|---|---|
| Alapa Market | 4.33 |
| Ipata Market | 2.33 |
| Ganmo Market | 19.67 |
| Share Market | 58.33 |
| Kaiama Market | 5.67 |
| Tsaragi Market | 3.00 |

**Table 2:** Percentage occurrence of fungi across various markets in Kwara State, North-Central Nigeria.

| Markets | *Aspergillus flavus* | *A. oryzae* | *Mucor* sp. | *Rhizopus arrhizus* | *R. oryzae* |
|---|---|---|---|---|---|
| Kaiama | 1 | 9 | 2 | 0 | 0 |
| Ganmo | 3 | 15 | 0 | 1 | 0 |
| Ipata | 1 | 1 | 2 | 1 | 0 |
| Tsaragi | 1 | 0 | 3 | 0 | 1 |
| Alapa | 1 | 1 | 1 | 1 | 0 |
| Share | 49 | 0 | 3 | 1 | 2 |
| Total | 56 | 26 | 11 | 4 | 3 |

**Table 3:** NCBI accession numbers and matched organisms.

| Organisms | Identification (%) | Query cover (%) | NCBI No. |
|---|---|---|---|
| *Aspergillus flavus* | 100 | 97 | PP735145 |
| *Aspergillus oryzae* | 99 | 99 | PP735146 |
| *Mucor indicus* | 98 | 98 | PP735147 |
| *Rhizopus arrhizus* | 98 | 100 | PP735148 |
| *Rhizopus oryzae* | 99 | 99 | PP735149 |

**Table 4:** Mean aflatoxin concentration of Peanut seeds from the 6 market places.

| Markets | Total-seeds tested | Positive (%) | Negative (%) | Mean-aflatoxin concentration (ppb) |
|---|---|---|---|---|
| Kaiama | 20 | 14 (70) | 6 (30) | 1.5 |
| Ganmo | 20 | 14 (70) | 6 (30) | 1.5 |
| Ipata | 20 | 11 (55) | 9 (45) | 1.0 |
| Tsaragi | 20 | 12 (60) | 8 (40) | 1.1 |
| Alapa | 20 | 11 (55) | 9 (45) | 0.9 |
| Share | 20 | 17 (85) | 3 (15) | 2.5 |
| Average | 120 | 79 (66) | 41 (34) | 8.5 |

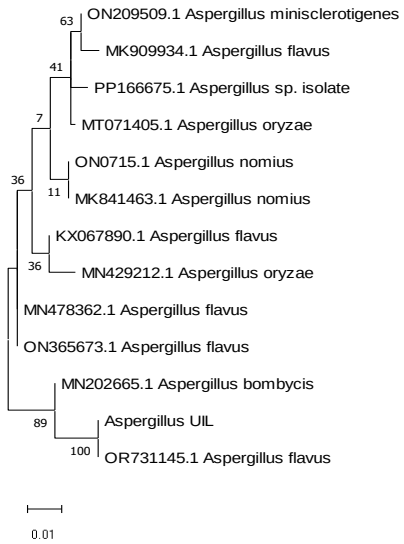


**Fig. 1:** Molecular phylogenetic analysis of *Aspergillus flavus* by Maximum Likelihood.

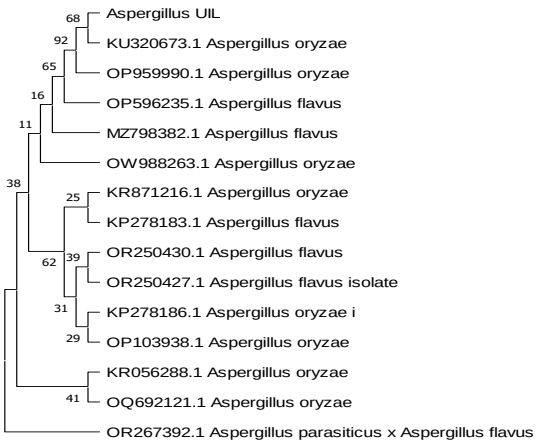


**Fig. 2:** Molecular phylogenetic analysis of *Aspergillus oryzae* by Maximum Likelihood.

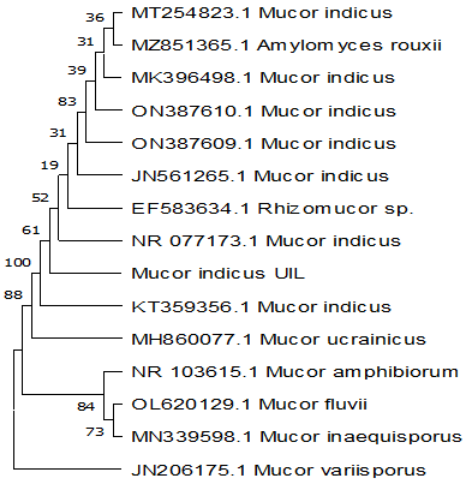


**Fig. 3:** Molecular phylogenetic analysis of *Mucor indicus* by Maximum Likelihood.

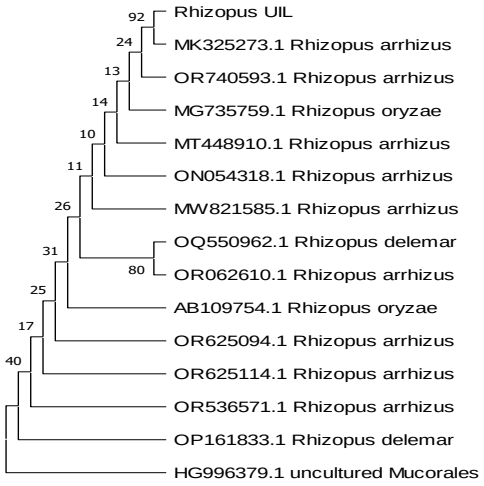


**Fig. 4:** Molecular phylogenetic analysis of *Rhizopus arrhizus* by Maximum Likelihood.

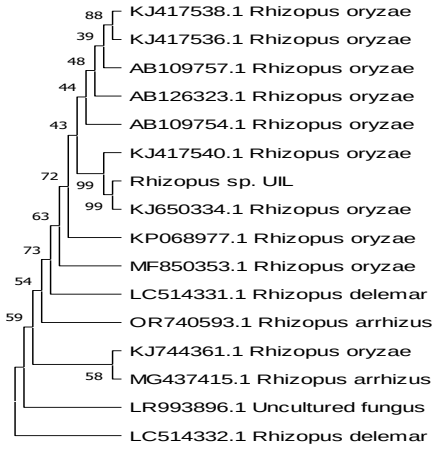


**Fig. 5:** Molecular phylogenetic analysis of *Rhizopus oryzae* by Maximum Likelihood.

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