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Research Article

Isolation and Phenotypic Identification of Mycotoxigenic Fungi from Stored Maize Kernels within Dutsin–Ma Metropolis, Katsina State

*** Ibrahim Salihu Salihu[1], Kilani Adetunji Musbau[1], Kamala Abdullahi[1], Hafsat Sagir Bakori[1], Abdul Nasir Aliyu[1], Sani Ahmad Shehu[2], Innocent John Wata[1]**

[1]Department of Microbiology, Federal University Dutsin-Ma, P.M.B 5001, Dutsin-Ma, Katsina State
[2]Department of Biochemistry, Faculty of Natural and Applied Sciences, Umaru Musa Yar'Adua University P.M.B 2218, Katsina, Nigeria
****Corresponding Author's email*: salihuibrahim299@gmail.com**

ABSTRACT

One of the most important effects of post-harvest decay or seed deterioration by fungi is the production of mycotoxins. Mycotoxicosis is a disease of animals and humans following consumption of feeds and foods contaminated by fungi that produce toxic substances called mycotoxins. The study investigated the cultural morphology, microscopic identification, pathogenicity, and mycotoxin production of fungal isolates from 25 stored maize samples collected. Standard microbiological techniques were employed for the analysis of all the samples, Cultural morphology on Potatoes Dextrose Agar revealed diverse colony characteristics, ranging from olive and dark green hues with smooth or spongy surfaces to brownish-pink and dull yellow-green colonies. Microscopic analysis identified six different fungal species, with *Aspergillus* spp being the most prevalent (72%) and *Mucor spp* as the least prevalent (6%). Pathogenicity tests on fresh maize demonstrated that all the isolates were pathogenic, causing observable growth on the maize samples. Furthermore, mycotoxin production by the fungal was revealed by evidence of fluorescence under UV radiation, indicating the potential production of aflatoxins. Stored maize kernels from the study area were at risk of fungal attack due to poor storage facilities, poor handling practices and inadequate drying of the grains, which enabled the fungi to grow and produce mycotoxins. Therefore, farmers should be sensitized on the possible risk of fungal attack on grains and be educated on timely harvest, hygienic drying and storage practices of grains.

Keywords: Mycotoxins, Maize, Mycotoxigenic Fungi, Fungal Isolates

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INTRODUCTION

One of the most important effects of post-harvest decays or seed deterioration by fungi is the production of mycotoxins. Mycotoxicosis is a disease of animals and humans following consumption of feeds and foods contaminated by fungi that produce toxic substances called mycotoxins (Tripathi and Alam, 2020). Common mycotoxicosis caused by common and wide spread fungi such as *Aspergillus, Penicillium, Fusarium* and *Stachybotrys* result in severe illness and deaths. *Aspergillus* and *Penicillium* produce their toxins mostly in stored seeds, hay or commercially processed foods and feeds, although infection of seeds usually takes place in the field (Adejumo and Adejoro, 2014). Fungal attacks by *Aspergillus, Penicillium, Rhizopus* and *Mucor* on stored grains occur after harvest and continue to grow on them during storage (Amadi and Adeniyi, 2009).

The genera of mycotoxigenic fungi are mainly represented by *Aspergillus, Penicillium* and *Fusarium,* but *Alternaria* among others are also important as

food contaminants or pathogens of plants. *Alternaria* produces mycotoxins in grains such as rice and maize (Ramm *et al.,* 2000). Many *Aspergillus, Penicillium, Fusarium* spp and *Cladosporium* species are known to produce mycotoxins, as the most important mycotoxin-producing fungi on maize. They produce mycotoxins such as aflatoxins, fumonisin, ochratoxins, deoxynivalenol, trichothecenes and zearalenone which cause significant economic losses in crops (Nooh *et al*., 2013; Darwish *et al*., 2014). However, according to according to Dooley (2001), other toxigenic fungi frequently found on grains are *Alternaria, Trichoderma, Fusarium, Paecilomyces, Chaetomium* and *Acremonium*. Mycotoxins are secondary metabolites produced by filamentous fungi which may contaminate food, feeds or raw materials used in producing them.

Aflatoxin is the most popular and widespread mycotoxin. Its name was derived from the fact that it was originally found to be produced by *Aspergillus flavus* (Tripathi and Alam, 2020), but is now known to be produced by other species of *Aspergillus*. Aflatoxin B1 is produced by *Aspergillus terreus,* though it may also be produced by *Aspergillus flavus,* as well as *Aspergillus oryzae.* It is the most toxic, carcinogenic and most prevalent of the different aflatoxins. Generally, mycotoxins have been implicated as causative agents of different human and animal health disorders. Both the toxigenic fungi and the mycotoxins they produce are potential problems from both health and economic perspectives (Dooley, 2001).

Maize (*Zea mays* L.) is an important cereal crop in the world, serving different purposes of economic significance (Chilaka *et al*., 2012). In Sub-Saharan Africa maize acts as a source of food and revenue to over 300 million households (Tefera *et al*., 2011) and constitutes an important source of carbohydrates, proteins, vitamins and minerals (Makone *et al*., 2014). Maize contamination by mycotoxigenic fungi is a major challenge worldwide (Lewis *et al*., 2005) and may occur in the field prior to harvest, at harvest, or at postharvest during storage (McMullin *et al*., 2015). Conventional methods employed in management of mycotoxins such as modifications in cultural practices, use of chemical fungicides and development of resistant cultivars (Palumbo *et al*., 2008) have only achieved minimal reduction in mycotoxin levels. The traditional storage methods such as use of granaries, gunny bags, polypropylene bags, plastic containers, open cribs (Wambugu, 2009; Gitonga *et al*., 2015) have been adopted by farmers. The extensive use of these conventional storage practices by smallholder farmers results in considerable post-harvest grain losses as a result of fungal growth and mycotoxin contamination (Bankole *et al*., 2006) which warrant investigations into finding appropriate storage technologies. Maize is an essential food crop. It is also used for animal feed production and as a source of income to farmers in Dutsin-Ma metropolis and its environs. However, contamination of stored maize with mycotoxins has become a serious problem in the area, which necessitated the focus of this research.

MATERIALS AND METHODS

Study Area

This research was carried out in Dutsin-Ma Local Government Area of Katsina state, Nigeria

Collection of Samples

Samples of stored maize kernels were collected from the different areas within Dutsin-Ma and environs. Samples were also obtained from agricultural commodity markets where the local vendors confirmed they purchased maize from. The samples were collected into clean sterile nylon bags, properly labeled and taken to the laboratory for analysis at the Department of Microbiology, Federal University, Dutsin-Ma.

Preparation of Culture Media and Inoculation

Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) were prepared according the manufacturers' instructions. The required amount of each medium was dissolved in distilled water followed by heating. Autoclaving was done in other to ensure sterilization. The sterile medium was then allowed to cool and dispensed into sterile Petri dishes. Potato Dextrose Agar (PDA) was used for growth and isolation of the fungal species. Kernels from the sub-sample were first washed with hypochlorite (2%) for 2 min and left to dry on sterile filter paper. The dried kernels were directly plated a minimum of three kernels per PDA plate, followed by incubation at room temperature (21 -25 °C) for seven days. The plates were inspected visually for fungal growth. The fungal isolates were sub-cultured at 25 °C for seven days to obtain pure isolates, which were used further analysis.

Identification of Fungal Isolates

Colony Characterization

The isolates were identified on the basis of cultural morphology and characteristic which included texture, pigmentation, powdery or fuzzy appearance, rhizoid or filament, and presence of fungal hyphae.

Lacto Phenol Cotton Blue Test

Lactophenol cotton blue test was conducted as demonstrated by Leck (2002). Drops of lactophenol were placed on a clean grease free slide. A portion of

the growths was picked using a sterile straight wire loop and gently teased on the lactophenol using two sterile inoculating pins. The preparation was then covered with a clean coverslip and examined under 40× and 10× objective lenses of the compound light microscope. Microscopic features used to identify the species of fungi included the hyphae (either septate or not), arrangement of micro-conidia, macro-conidia and chlamydospore (present or absent) and compared with mycological atlas.

Pathogenicity Test

Pathogenicity test was conducted as demonstrated by Barth and Zhesang (2009). Healthy matured maize (corn) was washed and surface sterilized with 70% alcohol. A sterile cork borer of 4mm diameter was used in punching out single corn, 4mm disc of fungal mycelia punched from pure culture of each of the isolates using sterile cork borer, each disc was then removed using sterile inoculating loop or needle and placed on the punched out space, one disc of fungal mycelia from one of healthy maize each. The inoculated points were covered with sterilized masking tape and then sealed up to prevent any cross contamination by other organisms. All the inoculated maize samples were incubated at 37^{o}C for 24hr. The pathogens were re-isolated from the inoculated maize samples; this was done by cutting out some portions of the spoiled maize samples with sterile scalpel and inoculating them directly onto sterile Sabouraud Dextrose Agar plates. The resultant growths were sub-cultured to obtain pure culture of the isolates which were re-examined macroscopically and microscopically.

Detection of Aflatoxins

Screening for aflatoxin production was done using colony fluorescence upon exposure to UV radiation at 365 nm. The sub-cultured plates were placed on reverse side of each Petri dish under ultraviolet radiation. Fungal colonies showing fluorescence upon exposure to UV radiation had the ability to produce aflatoxins like AFB1, AFB2, AFG1 and AFG2 (Liao *et al*., 2011) are grouped based on their fluorescence under blue and green UV light (Womack *et al*., 2013).

RESULTS

Cultural and Microscopic Characteristics of the Fungal Isolates

The investigation carried out showed various colonial morphologies of different fungal species on Potatoes Dextrose Agar. Most of the growths on the plates produced olive and dark greenish colorations, smooth with white edges and spongy surfaces; others had fluffy surfaces with black colorations. In addition, other produced brownish-pink colonies, pale, dull yellow and green colonies. The microscopic examination revealed the presumptive identities of the isolates. Six different fungal species were recovered. The results are presented in table 1.

Frequency Distribution of Fungal Isolates from the Various Sampling Locations

Figure 1 illustrates the distribution of fungal isolates obtained from various sampling locations. Hayin Gada had *Aspergillus spp* (40%) and Fusarium spp (20%); while Abuja Road area recorded *Aspergillus spp* (40%), *Fusarium spp* (20%) and *Mucor spp* (20%).

Percentage Occurrence of Fungal Species on Maize Samples from the Study Area

The most prevalent fungus was *Aspergillus spp* (72%), while the least occurring was *Mucor spp* having 6 as percentage of occurrence. The results are shown in Figure 2.

Pathogenicity of the Fungi Isolates

After inoculating the fungal spores onto mature fresh maize samples, all the isolates were able to infect the maize, hence pathogenic.

Mycotoxins Detection

Out of the total of 15 isolates screened, only 5 isolates developed pink colour pigmentation upon contact with ammonium vapours and characteristic blue fluorescence under long wavelength (365 nm) of UV light indicating their ability to produce mycotoxins (Figure 3).

Table 1: Macroscopic and Microscopic Characteristics of Presumptive Fungal isolates

Sample code	Colonial morphology	Microscopic characteristic	Presumptive isolate
HG1	Olive green granular surfaces colonies	Thick-walled conidiospores with hyaline and long aseptate shape	*Aspergillus flavus*
HG2	Brown or pink in center with white surfaces	Microconidia, long phialide and chlamydospores	*Fusarium spp*
HG3	Black-white edges and spongy surfaces	long conidiospores with smooth walled hyaline	*Aspergillus niger*
DK1	Dark green-white edges and spongy surface	Dark-narrow conidiospores with walled hyalines	*Aspergillus fumigatus*
DK2	Black -white edges and spongy surfaces	long conidiospores with smooth walled hyaline	*Aspergillus niger*

WD1	Pale -dull yellow -green colony	Long and large conidiospores roughly in distal part, thick wall	*Aspergillus oryzae*
WD2	Olive green granular surfaces colonies	Thick-walled conidiospores with hyaline and long aseptate shape	*Aspergillus flavus*
WD3	Dark-green rough surface colonies	Conidia head and conidiospores that are short	A*spergillus parasiticus*
AR1	Brown or pink center with white surfaces	Microconidia, long phialide and chlamydospores	*Fusarium spp*
AR2	Cotton like white growth with black spots	Sporangia with spores, no rhizoids	*Mucor spp*
AR3	Dark green colonies with rough surfaces	Conidia head and short conidiospores	A*spergillus parasiticus*
AR4	Dark green, white edges and spongy surfaces	Dark-narrow conidiospores with walled hyalines	*Aspergillus fumigates*
DW1	Olive green white granular surfaces	Thick-walled conidiospores with hyaline and long aseptate shape	*Aspergillus flavus*
DW2	Black-white edges and spongy surfaces	long conidiospores with smooth walled hyaline	*Aspergillus niger*
DW3	Brown or pink in center with white surfaces	Microconidia, long phialide and chlamydospores	*Fusarium spp*
KD1	Dark green, white edges and spongy surfaces	Dark-narrow conidiospores with walled hyalines	*Aspergillus fumigatus*
KD2	Brown-pink center with white surfaces	Microconidia, long phialide and chlamydospores	*fusarium spp*
KD3	Dark green colony color, rough surfaces	Conidia head and conidiospores that are short	A*spergillus parasiticus*

Key: *HG = Hayin Gada, WD = Wednesday market, AR = Abuja Road, DW = Darawa, KD = Kadangaru*

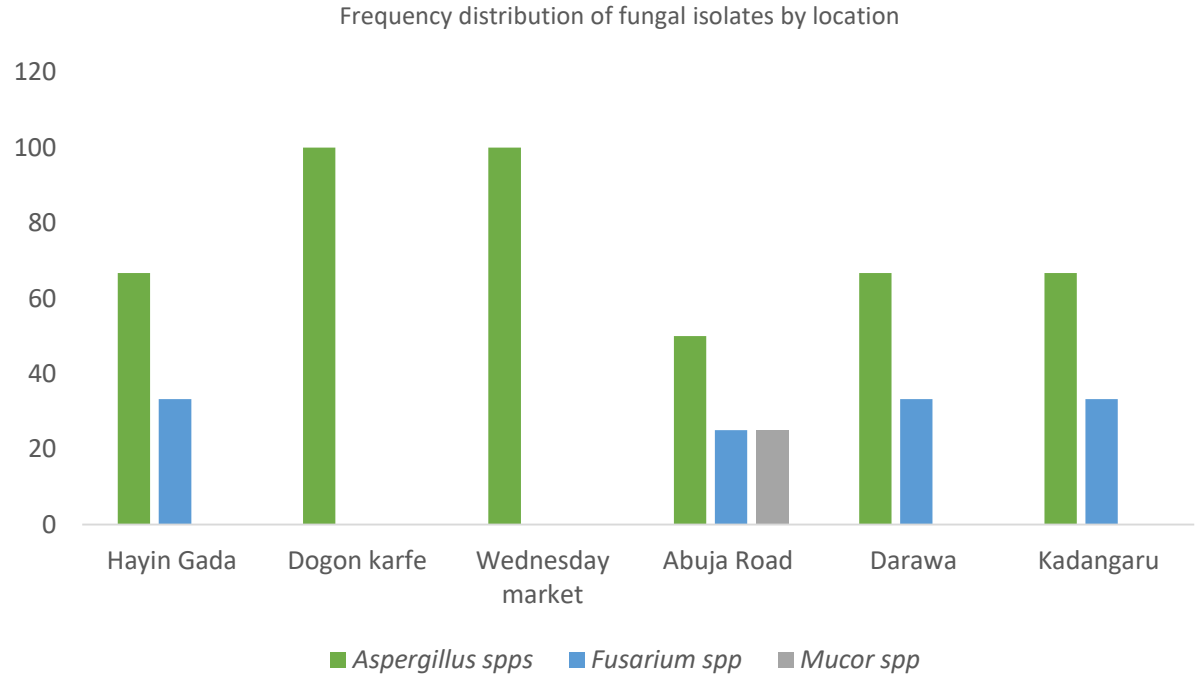

Figure 1: Frequency Distribution of Fungal Isolates from the Various Sampling Locations

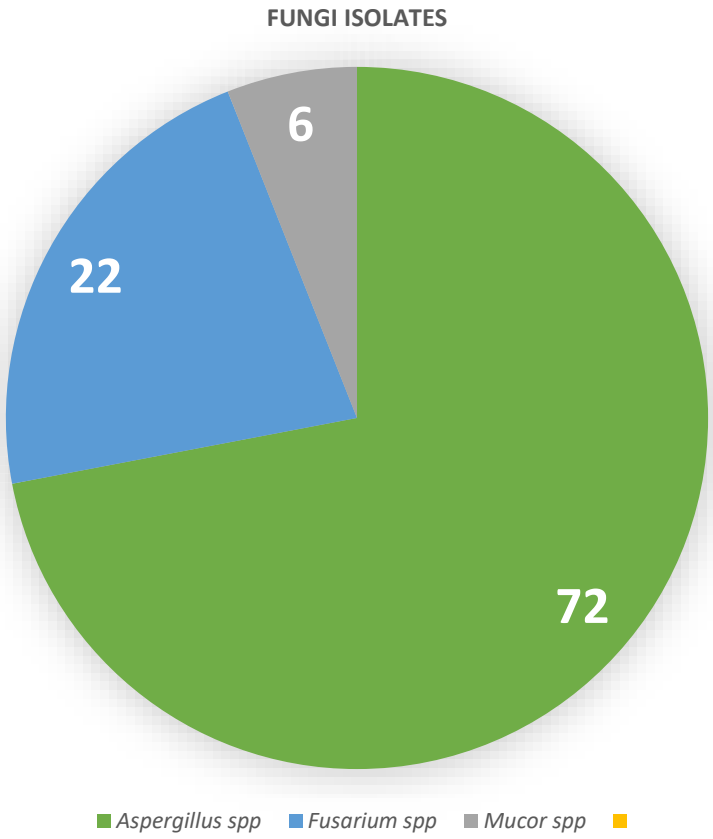

Figure 2: Percentage Occurrence of Fungal Species on Maize Samples from the Study Area

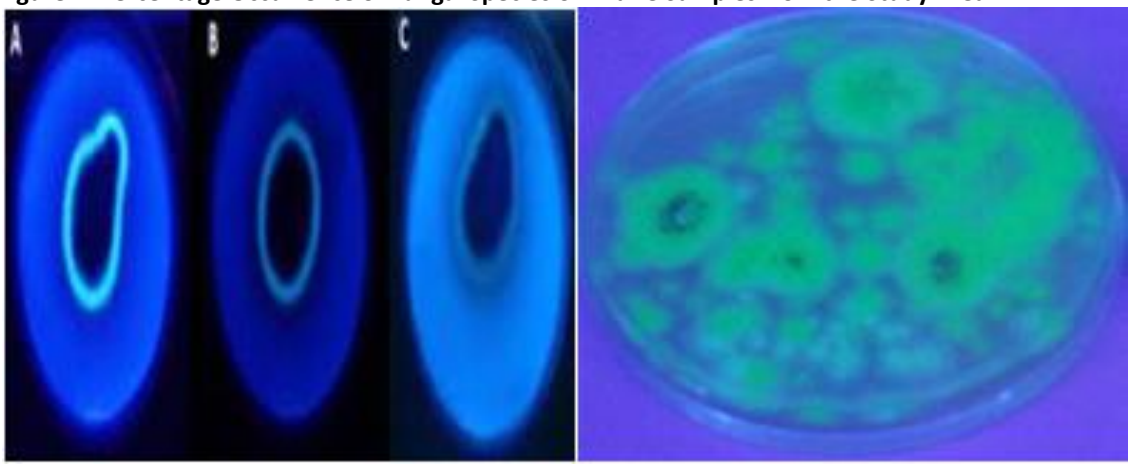

Figure 3: Detection of Aflatoxin Production by Fungal Isolates upon Exposure to Ultraviolet Light

Table 2: Pathogenicity Test Results on fresh matured Maize

Fungal isolate inoculated	Observation
Aspergillus niger	Black powdery/foamy growth
Aspergillus parasiticus	Greenish powdery growth
Aspergillus fumigatus	Greenish powdery growth
Fusarium spp	Whitish powdery growth
Aspergillus oryzae	Yellowish powdery growth

Figure 4: Pictorial presentations of pathogenicity property of the isolated fungal species

DISCUSSION

Poor storage facilities, poor handling practice and inadequate drying of maize predispose it to attack by fungi capable of producing mycotoxins in them. Various fungal species were isolated from stored maize kernels within the study area. *Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus,* and *Fusarium spp were* among the four different fungi isolates that were identified. These fungal isolates were found to produce aflatoxins on fresh maize samples, which could cause food poisoning. In this study *Aspergillus* spp was the most commonly isolated fungal pathogen from maize samples, followed by *Fusarium* spp*.* Predominance of *Aspergillus spp* in maize sample agrees with the findings by Bii *et al*. (2012*)* who reported that the most frequently isolated fungal genera from maize were *Aspergillus* (35.8%), followed by *Fusarium* (15.5%) then *Penicillium* (9.2%) and *Rhizopus* (5.3 %); while the incidence of other fungal species was 34.4%. This research also disagrees with the findings of Muthomi *et al*. (2009) who also identified *Fusarium* spp as the most commonly isolated fungal species in maize. High diversity of mycotoxigenic fungi in maize grains poses a health risk of exposing consumers of maize and maize products to mycotoxins (Wagacha *et al*., 2013). These findings are in agreement with the report by Tsedaley and Adugna (2016) that the populations of *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. were higher in stored maize samples compared to maize samples collected at harvest. The composition of fungal species established in the field and effects of rain prior to harvest or during storage significantly influence the development of mycotoxigenic fungi during storage (Hirooka *et al*., 2007). However, a study by Murithi (2014) isolated *A. flavus* L- strain, *A. flavus* S-strain and *A. parasiticus* in maize. Other studies on maize microflora by Nyukuri (2007) reported that *A. parasiticus, A. flavus*, *A*. *niger* as well as other *Aspergillus* spp were isolated from harvested maize grain samples. Similar spectrum of *Aspergillus* spp reported at harvest was also observed in maize grains sampled three months after storage in polypropylene and hermetic bags. This could be explained by the occurrence of corresponding high population of *Aspergillus* spp resident in maize sampled at harvest which influences the population in storage. In this study, the population of *Aspergillus* spp in maize was dominant from the stored maize during storage. This agrees with reports by Wagacha *et al*. (2013) that the population of *A. flavus* and *A. parasiticus* progressively increased during storage and was significantly higher at the third month of storage. Domenico *et al*. (2016) reported that there was an increase in the population of *Aspergillus* spp. in maize after three months of storage with a progressive increase until nine months. A previous study by Hell *et al*. (2003) also observed higher frequencies of *A. flavus* in stored maize compared to maize obtained at harvest. All isolates of were noted to be pathogenic on corn by carrying out a pathogenicity test with the fungal isolates. The study of disease control is crucial since the pathogens are able to produce harmful mycotoxins. This agrees with the findings of Zainudin *et al.* (2017) who carried out pathogenicity of fungal isolates on corn sample.

CONCLUSION

Maize production practice by farmers in Dutsin-Ma metropolis is at risk of fungal attack and mycotoxin contamination. Lack of timely harvesting and improper storage practices also predisposed the maize to fungal attack which produced mycotoxin on them. *Aspergillus* spp and *Fusarium* spp were the major mycotoxin-producing fungi isolated from maize grains sampled at storage

Findings of this research showed that there is need for proper training of farmers on all processes that involved maize cultivation, drying and storage. Maize drying should be at ≤ 13% moisture content before storage. Storage of maize in appropriate storage facilities that are well ventilated should be encouraged. Use of modern granary reduces the chances of maize contamination with mycotoxin-producing fungi.

Conflict of Interest

The authors declare no existing conflict of interest.

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