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Regulatory Gene, *aflaR* Identification of Aflatoxigenic Moulds and total Aflatoxin estimation in *Digitaria exilis* and *Digitaria iburua* sold within Kaduna Metropolis

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Abstract: *Contamination of Digitaria sp by aflatoxigenic strains could pose a serious health threat to food safety. In this study, a design is made with the aim of screening and characterizingmoulds isolated from Digitaria iburua and Digitaria exilis sold within the Kaduna metropolis. Seventy samples of Digitaria sp were obtained from four outlets of some markets within the Kaduna metropolis. The proximate values were determined following standard procedures. Isolation and characterization of the moulds were done using standard techniques. Polymorphic technique and regulatory gene aflaR were employed to identify the aflatoxigenic moulds isolated from the samples. Analysis of the aflatoxin content of the samples was carried out using Enzyme-Linked Immunosorbent Assay (ELISA) technique. This research has recorded proximate values determined for Digitaria sp as follows: moisture content 6.90%, protein 11.10 %, ash 3.90 %, fats 3.9 %, and carbohydrate content 74.16 % for Digitaria iburua. Digitaria exilis presented a moisture content of 7.3 % while other proximate contents were protein (10.2%), ash (3.21%), fats (2.3%), and carbohydrate content (76.99%) %. The moulds; Aspergillus flavus, Rhizopus stolinifer, Fusarium sp, Saccharomyces cerevisae, Aspergillus parasiticus, Aspergillus niger, mucor sp and Penicillium sp were isolated from both Digitaria exilis and Digitaria exilis with Aspergillus flavus having the highest frequency of occurrence. The total viable mould count for each sample location ranged from $1.5X10^3$ – $2.8X10^3$ CFU/g, $2.3X10^3$-$3.1X10^3$ CFU/g, $2.6X10^3$-$5.4X10^3$ for Kawo, Sabo and Central markets respectively. Analysis of the total aflatoxin content of the seventy samples showed a prevalence of 16.7 %. There is a need to sensitize farmers and consumers on the occurrence of the toxin and also the need to develop a resistant variety is hereby advocated.*

Keywords: *Aflatoxigenic moulds, aflaR, aflatoxins, carcinogen, Digitaria sp*

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1.0 Introduction

Digitaria sp is a very small cereal that belongs to the grass family Digitaria. Commonly within the study area, there are two types of *Digitaria* sp including brown color (*Digitaria iburua*) and white coloured (*Digitaria exilis*). Both species are called Hausa *Acha*, It is consumed as a cereal and remains an important crop in Eastern Senegal, Western Burkinafaso, Southern Mali and Northern Nigeria (Cruz, 2004). The cereal has high economic value. *Digitaria* sp is consumed across northern Nigeria and neighboring countries. The cereal serves as a staple food in the form of porridge and also beverages. The cereals contain high amino acids and mineral contents (Nzelibe *et al.,* 2000).

Pre-harvest contamination of the cereals by aflatoxigenic moulds (e.g. *Aspergillus* sp) poses a serious threat to food safety due to the liberation of toxic secondary metabolites such as aflatoxins (Cotty, 1994; Wild, 2007; Somorin *et al*., 2010). Aflatoxins also affect cereals during the post-harvest stage due to inadequate drying and storage under highly humid conditions (Jimoh *et al.,* 2008). Aflatoxins are naturally occurring mycotoxins produced fundamentally by *Aspergillus flavus* and *Aspergillus parasiticus* (Gbodi *et al.,* 2004; Dorner *et al.,* 2007; Ezekiel *et al.,* 2012). They are the most important group of mycotoxins because of their severe public health carcinogenic associated risks *(*IARC, 1993*;* Cotty, 1994; Bankole *et al.,* 2006). Aflatoxin exposure could lead to impaired growth, hyperplasia of the bile duct, immune suppression, hepatocellular carcinoma, etc (Williams *et al*., 2004; Wild, 2007; Umoh *et al*., 2011).

Molecular techniques have been used to detect aflatoxigenic moulds. The primer *aflR* is that of DNA segment of the aflatoxigenic strain that regulates key structural genes (*omt-A, ver-1* and *nor-1*) at the transcription level in the polyketides pathway of aflatoxin biosynthesis (Schem *et al*., 2005). Therefore, this research aimed to use cultural and *aflaR* genes to identify aflatoxigenic moulds and also to estimate the total aflatoxin content of *Digitaria* sp sold within the Kaduna metropolis.

2.0 Materials and Methods

2.1 Sample area

A Survey of the incidence of aflatoxins and aflatoxigenic moulds in *Digitaria iburua* and *Digitaria exilis* was conducted within the Kaduna metropolis. The samples were bought from some major markets within the metropolis i.e. Kawo, Central, Sabo and Kakuri markets.

2.2 Sample collection

Digitaria sp was purchased from the various sales market outlets within the metropolis. The grain mass was mixed to form a homogenate sample before aseptically collecting using a sterile spoon into sterile low-density cellophane bags and transported to the Department of Microbiology laboratory. A total of seventy samples was collected from the four major markets once every week from two major distribution for each market.

2.3 Determination of the proximate composition of Digitaria iburua and Digitaria exilis

Proximate values (percentage moisture, ash, protein, fats and carbohydrate contents) were determined following standard procedures of the Association of Official Analytical Chemists (AOAC) 1984.

2.4 Isolation of fungi in Digitaria iburua and Digitaria exilis

A stock solution of the sample was prepared by weighing twenty-five (25) grams each of *Digitaria exilis* and *Digitaria iburua* separately. This was poured into a sterile 500

mL capacity conical flask containing 225 mL of sterile peptone water. The mixture was sealed and stirred for five minutes. Then, 1.0 mL was withdrawn using a sterile pipette from the stock solution into a test tube containing 9.0 mL of sterile peptone water and was mixed. Then fivefold serial dilution was made. From each dilution, 1.0 mL was pour plated with 20 mL of sterile sabouraud dextrose agar supplemented with 0.5g/l Chloramphenicol. The culture plates were allowed to solidify and incubated at 27 ± 2^{o}C for 7 days. The culture was characterized following the standard method of Klich (2002) adopted by Wartu *et al*. (2019).

2.5 Molecular identification of aflatoxigenic strain using aflaR

Moulds were first cultured using specific detection media, Yeast extract sucrose agar (YESA) modified with 0.3% cyclodextrin and 0.6% sodium desoxycholate (YCSD) according to the method described by Ordaz *et al*. (2003). Then the pure cultures of the isolates that presented a beige ring were used for the identification of aflatoxigenic strain using *aflaR* according to the methods adopted by Wartu *et al. (*2017) using the primer *aflaR*-F 5`-TAT CTC CCC CCG GGC ATC TCCCGG-3`, R5`- CCG TCA GAC AGC CAC TGG ACACGG-3` with target amplicon size of 1032bp (Shem *et al*., 2005).

2.6 Determination of aflatoxin content of Digitaria iburua and Digitaria exilis

The analysis of the aflatoxin content of the samples was determined using Enzyme Linked Immune-sorbent Assay (ELISA) following the manufacturer's (Helica Biosystems, USA) instructions adopted by Wartu *et al*. (2015).

3.0 Results and Discussion

The mean proximate values determined for *Digitaria exilis* are shown in Table 1. The two varieties of *Digitaria* sp have varying proximate values as determined. The moisture content for both species was very low (6.90 and 7.30%) for *Digitaria iburua and Digitaria exilis* respectively. Protein and fat contents were higher for *Digitaria iburua* than *Digitaria exilis* Table 1.

The mold counts (CFU/g) and the occurrence of moulds isolated from *Digitaria* sp are presented in Tables 2 and 3 respectively. Samples from the Central market were seen to have a higher mean mould count (3.76×10^3CFU/g) while samples from the Kawo market had the lowest mean mold count (2.12×10^3 CFU/g). However, there was no significant difference ($p\leq 0.005$) between the two market moulds counts

Table 1: Proximate composition of Brown and White *Digitaria* sp

Parameter (%)	Brown (*Digitaria iburua*)	White (*Digitaria exilis*)
Moisture	6.90	7.30
Ash	3.90	3.21
Protein	11.10	10.20
Fats	3.94	2.30
Carbohydrate	74.16	76.99

*****Mean of tripkecate analysis**

Table 2: Mean Total viable mould count of *Digitaria* sp

Sample location	Brown (*Digitaria iburua*)	White (*Digitaria exilis*)
K	2.1×10^{2a}	3.9×10^{2b}
S	2.7×10^{2a}	2.1×10^{2a}
C	3.6×10^{2b}	$2.9 \times 10^{2\,a}$
KK	2.8×10^{2a}	2.7×10^{3a}

***Mean of triplicate analysis, Values across the row and column with the same superscripts do not vary significantly at $P \leq 0.05$

Key: K= Kawo market, S= Sabo market, C= Central market, KK=Kakuri

The moulds; *Aspergillus flavus, Rhizopus stolinifer, Fusarium* sp, *Saccharomycetes cerevisae, Aspergillus parasiticus, Aspergillus*

niger, mucor sp and *Penicillium* sp. were isolated from *Digitaria* sp (Table 4). All the samples showed varying levels of moulds and yeast. The moulds *Aspergillus flavus* have the highest frequency of occurrence followed by *Fusarium* sp in this study.

The aflatoxin content determined from *Digitaria* sp is shown in Table 5. The level of aflatoxin contamination was higher for samples from the Sabo market having a mean concentration of 4.65 µg/Kg, while samples from the Central market had the lowest mean concentration of 1.54 µg/Kg. The detection of the total aflatoxin content in the samples showed that all the samples had aflatoxin contamination ranging from 0.6 - 2.9 µg/Kg for Kawo market samples, 2.6-9.6 µg/Kg for Sabo market, while that of Central market ranged from 0.4-3.6 µg/Kg. The total aflatoxin content of the samples showed a prevalence of 16.7 %. There was an amplification of the gene *aflaR* using PCR at 1032bp (Plate 1).

Table 3: Morphological and microscopic characteristics of moulds isolated from *Digitaria sp.*

Colony morphology	Microscopic morphology	Probable organism
Black colonies.	Septate hyphae, long conidiophores.	*Aspergillus niger*
Colonies are initially white with colorless to dark purple reverse	Hyphae are septate and hyaline conidiospores are medium-length. Macro conidia are septet very slightly sickle-shaped to nearly straight	*Fusaruim* sp
Dark green colonies.	conidiophores are short with small vesicles attached to the phialides	*Aspergillus parasiticus*
Colonies produced a fluffy white growth.	Non-septate hyphae, sporangiophores are long with spores around the sporangiophores.	*Mucor* sp
White and creamy cotton-like mycelia Cottony white	Non-septate, oval-shaped with short conidiophores. Bean shaped	*Rhizopus* sp
Green colonies which became powdery.	Septate hyphae and produced brush-like conidiophores.	*Penicillium* sp
A powdery mass of yellow-green spores then later became dark green.	Branched hyphae, with a radiant conidial head	*Aspergillus flavus*

Table 4: Occurrence of *Aspergilus flavus* and *Aspergilus parasiticus* isolated from *Digitaria* sp

Samples	*Aspergilus flavus*	*Aspergilus parasiticus*
K	70.0	30.0
S	60.0	40.0
C	80.0	20.0
D	65.0	35.0
KK	45.0	55.0

**** Means of duplicate analysis, K= Kawo market, S = Sabo market, C = Central mosque, KK = Kakuri**

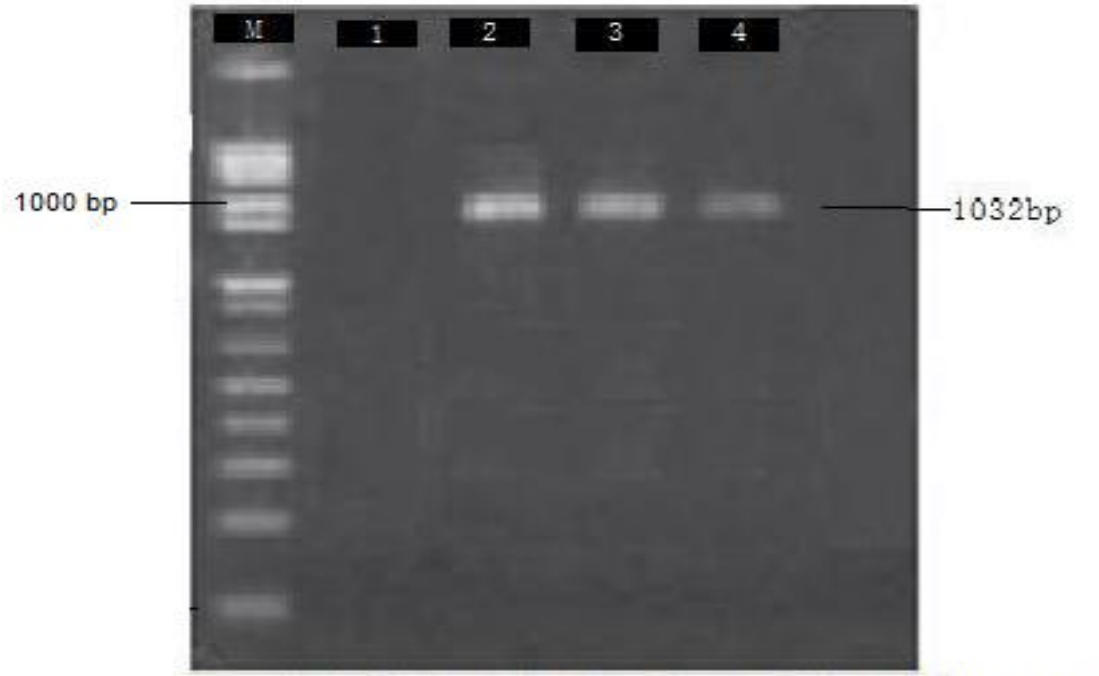

Fig. 1: Gel picture sowing PCR products (2, 3, 4) , 1, -ve control primer aflaR from aflatoxigenic Aspergillus sp.
M, 100bp lader. , 1, 2, 3 & 4 are sample wells

Table 5: Mean Aflatoxin Content Determined from *Digitaria exilis and Digitaria iburua*

Sample	Mean concentration (µg/Kg)	
	Brown (*Digitaria exilis*)	white (*Digitaria iburua*)
K	1.80	2.00
S	4.60	3.70
C	1.50	1.60
D	1.90	2.80
KK	2.50	1.00

*****Mean of triplicate analysis, K = Kawo market, S= Sabo market and C= Central Market KK, Kakuri**

Proximate values of *Digitaria iburua and Digitaria were determined.* The percentage of protein and fat content obtained in this research is lower than the reported values by Ogbonnaya (2009). The differences could be due to different analytical procedures, sources and or soil types where the cereals were cultivated. The nutritional benefit of *Digitaria* sp is evident because of its high protein content and thus could serve as a complement to standard diets.

The difference in the level of mold contamination could be due to the storage of the cereal under conditions favorable for the development of the moulds. Earlier, other researchers have reported the contamination of grains right from the farm (Scheidegger and Payne, 2012). Likewise, when cereals are stored under high moisture and favorable temperature, moulds and aflatoxin development could occur (Smith and Moss, 1985). At the time of sampling, the *Digitaria* the temperature which is usually room temperature (27± 2^oC). This temperature favors the growth of moulds and when aflatoxigenic strains are present, they produce aflatoxins when environmental conditions are favorable (David and Jones, 2008).

The implication of the aflatoxigenic *Aspergillus flavus* and *Aspergillus parasiticus* in foods is that they serve as an important tool in assessing the toxicological status of food because they are capable of producing aflatoxin B_1, B_2, G_1, G_2. The moulds isolated from this study were either storage or field fungi especially the field moulds *Fusarium* sp and *penicillium* sp require high moisture levels (18.0 and 16.0 – 19.0 %) respectively than the storage fungi. Storage fungi require a relative humidity of at least 65% (or a water activity of $a_w = 0.65$) which is equivalent to an equilibrium moisture content of 13% in cereal grain. Earlier, Larry (1982) reported minimum a_w at which growth of fungi has been observed is about 0.61. Since the mean moisture levels for brown (6.90%) and white (7.30%) *Digitaria* sp were low (Table 1), the occurrence of moulds in the samples could be due to contamination when the grains were still wet and probably unharvested or poor drying technique or Delay in drying to safe moisture level as explained by (Archana and Manish, 2014). Most drying methods within the study area involve spreading the produce on mats and high-density cellophane to dry under natural sunlight. Since moulds are ubiquitous they contaminate the exposed sp were being sold in open pans and dirty sacks. In addition, some were seen marketed along dusty market foot pathways. This could be responsible for the contamination and hence the high count from this study. Earlier, the author David and Jones (2008) documented the contamination of grains by mould spores transported by air currents. Bullerman and Andrea (2011) reported that the sources of contamination of cereals are traceable to the storage condition and the produce and hence the occurrence and high count of the moulds from this research.

The regulatory gene *afla*R, regulates key structural genes in the polykitide pathway of aflatoxin biosynthesis. The structural gene *omt-A* encodes the enzyme O-methyltransferase A, which steps in the aflatoxin biosynthetic pathway through the conversion of Sterigmatocystin to O-methylsterigmatocystin. The primer *ver-1* is a sequence of a structural gene (versicolorin A) which encodes an enzyme that is directly involved in the conversion of Versicolor in A to demethylsterigmatocystin. The primer *nor-1* amplifies the norsolorinic acid gene that encodes an enzyme norsolorinic acid ketoreductase along the polyketide pathway (Yu *et al.*, 2004).

According to the European Commission, all aflatoxins must be absent in agricultural products for human consumption (European Commission, 2006). Aflatoxin content in some of the samples exceeded the National Agency for Food and Drugs Administration and Control (NAFDAC) limit of 4 µg/Kg for cereal. Thus, exposure of consumers to low or moderate levels of aflatoxins in crops does not make it entirely safe. Therefore, individuals consuming such contaminated crops at regular basis could be at risk of aflatoxicosis.

4.0 Conclusion

In conclusion, from this research, the proximate values for *Digitaria* sp were estimated. The research reveals higher nutritional content of *Digitaria iburua* than

Digitaria exilis. The moulds; *Aspergillus flavus, Rhizopus stolinifer, Fusarium* sp, *Saccharomycetes cerevisae, Aspergillus parasiticus, Aspergillus niger, mucor* sp and *Penicillium* sp. were isolated from *Digitaria* sp with *Aspergillus flavus* having the higher frequency of occurrence.

Analysis of the total aflatoxin content of the seventy samples showed a prevalence of 16.7 % Two (2.0) % of the samples had aflatoxin contamination exceeding the National Agency for Food and Drugs Administration and Control (NAFDAC) limit of 4 µg/Kg for cereal grains. There is a need to sensitize farmers and consumers on the risk factors associated with exposure to aflatoxins being a carcinogen.

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Consent for publication

Not Applicable

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