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Molecular Characterization of Mycotoxin Producing Fungi Contaminating Groundnut Products in Sokoto State, Nigeria

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ABSTRACT:

Groundnut products (kernel, cake and oil) are widely consumed among the people of Sokoto state, especially by children of school age and young adults. Groundnut products serve as supplementary protein source, and as ingredient in poultry feed formulation. A preserved isolates of mycotoxin producing fungi (*Aspergillus niger*, *A. flavus*, *A. fumigatus*) were collected from mycology lab of Usmanu Danfodiyo University, Sokoto. Samples were characterized through Gotaq PCR assay and RAPD analysis. Fourteen identified mold isolates were confirmed by PCR using primer pairs ITS1/ITS4, ITS1/NIG and ITS1/FLA. Random amplified polymorphic DNA (RAPD) analysis with eight primers revealed similarity in band patterns between the isolates and the control. Confirming the identification of potential Aflatoxin producing *Aspergillus flavus* and Ochratoxin producing *A. niger* strains in the groundnut products. The results obtained in this study pointed out that Aflatoxin gene cluster variability existing in populations can be useful for understanding the toxicological risk as well as the selection of biocontrol agents.

Keywords: *Molecular, Aflatoxin, Aspergillus, Groundnut products and Fungi.*

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INTRODUCTION

Fungi have traditionally been identified on the basis of taxonomic characteristics but more recently are classified through genotyping involving nucleic acid sequencing. Fungi exhibit greater species richness than most other organisms and, thus, are of significant environmental and economic importance [1]. Recent predictions based on molecular methods have suggested that there are 5.1 million fungal species [2]; however, only approximately 5% of the predicted filamentous fungal species have been described [3]. The mycotoxigenic fungi biosynthetic pathway involves approximately 25 genes clustered in a 70 kb DNA region [4]. *A. flavus*, *A. niger*, and other *Aspergillus* species share nearly identical sequences and conserved gene order in the cluster. In recent years PCR detection of mycotoxigenic fungi biosynthetic gene presence or expression has been used as diagnostic tool for mycotoxigenic fungi in selected food commodities [4]. Sequence variability and deletions in various regions of the mycotoxigenic biosynthetic cluster have also been used to determine the polyphyletic assemblage of *A. flavus* group [5, 6].

The use of PCR to identify mycotoxigenic fungi is attracting considerable attention [7]. These methods are based on genes separated from mycotoxin biosynthesis. However, there are only a few mycotoxins about which the biochemistry has been determined sufficiently to enable the development of gene probes of the pathway [8]. It is worth mentioning that many of the methods are intended ultimately to be used on ‘‘virgin’’ samples, i.e. those which otherwise have not been examined [9].

The random amplified polymorphic DNA (RAPD) technique has been used to characterize and detect genetic variability between isolates of *A. flavus* and related species. Most of the molecular methods rely on the determination of the DNA amplicon sequence and its sequence comparison with those available from GenBanks.

Mycotoxigenic fungi determination and identification up to molecular level remain a priority before establishment of any health implication and control system. Although many works were reported on the worldwide occurrence of mycotoxin-producing fungi in groundnut products and many other agricultural products, no work has been published on contamination of groundnut products with mycotoxin producing fungi in Sokoto state, as well as molecular characterization of such mycotoxigenic fungi. Therefore, it is against the background that the present study was embarked upon on Molecular characterization of mycotoxigenic fungi contaminating Groundnut product in Sokoto state, aimed at Genotypically characterized the Mycotoxin producing fungal isolates by PCR assay and Determine the Genetic similarities among the isolates through Random Amplification Polymorphic DNA (RAPD) Analysis.

MATERIALS AND METHODS

Collection of Samples

Samples were collected from the slanted bottles, preserved in mycology lab of Biological Sciences Department, Usmanu Danfodiyo University. They were *Aspergillus niger, Aspergillus flavus* and *Aspergillus fumigates*

Molecular Identification of mycotoxin Producing *Aspergillus* Strains

Isolates were purified for molecular identification at the species level. All *Aspergillus* sp. strains were identified.

DNA extraction

Total genomic DNA of isolates was extracted using a slight modification methods by Cenis[10]: 7 day-old mycelium

was gently scraped off with aseptic scapel from PDA vijue bottles, transferred directly into 1.5 ml Eppendorf tubes containing 400 µl of lysis buffer (200 mM Tris- HCL pH 8.5, 250 mM NaCl ,25 mM EDTA) and homogenized for 3 min using Eppendorf micro pestles (Eppendorf, Hamburg, Germany); 25 µl of 0.5% SDS were then added and tubes were placed at 65°C for 10 mins. After addition of 150 µl of 3M sodium acetate, the tubes were placed at – 20°C for 10 mins, centrifuged at 13,000rpm for 10 mins and the supernatant transferred to a new tube. The DNA was precipitated by adding an equal volume of isopropanol and left for at least 5 minutes at room temperature. After 10 minutes of centrifugation at 13,000rpm, the pellet was washed with 70% ethanol, dried at room temperature and resuspended in 100 µl TE-buffer.

PCR Amplification

PCR analysis was performed according to the method by Dachoupakan [11] with a slight modification using GoTaq PCR Core Systems I (Madison, WI, USA). ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') was used in combination with each of the following primers: NIG (5'-AGACAGGGGGACGGC-3') and FLA (5'-CGG CCC TTA AAT AGC CCG GTC-3'). A total PCR mixture of 25 µL containing: 14.75 µL deionized water, 2.5 µl of 5x buffer green, 2.5 µL of MgCl2 (25mM), 1 µL dNTP (10 pmol/µl), 1 µl of primer ITS1 (10pmol/ µL), 1 µl of primer NIG for *A. niger* or 1 µl of primer FLA (10 pmol/ µl) for *A. flavus*, 0.25 µL of Taq DNA polymerase and 2 µL of DNA template. PCR amplification
was performed in a Eppendorf Master Pro (Applied Biosystem, Califonia, USA) cycler and cycling conditions were as follows : 94°C for 3 min followed by 35 cycles at 94°C for 40 sec, 54°C for 40 sec, 72°C for 1 min and a final extension at 70°C for 10 min [11, 12].

Gel Electrophoresis

The PCR products were separated on 1.5 % (w/v) agarose (Bio-Rad, California, USA) stained with 0.8µg/ml ethidium bromide and visualized under ultraviolet (UV) photographed by Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA). A 1 Kb plus DNA ladder Gene ruler (Thermo Scientific, California, USA) was used as a standard marker to compare molecular masses of amplified DNA.

RAPD-PCR Analysis

Amplification of fungal DNA was performed according to the method described by Dachoupakan [11] with slight modifications using each of the following primers (Applied Biosystems, California, USA): OPC-03 (5'-TGGACCGGTG-3'), OPC-4 (5'-TGTCTGGGTG-3'), OPC-5 (5'-TGTCTGGGTG-3'), OPC-6 (5'-TGTCATCCCC-3'), OPC-7 (5'-AAGCCTCGTC 3'), and OPC-8 (5'-TGCGTGCTTG-3'). PCR was performed using GoTaq PCR Core Systems I (Madison, WI, USA) in a total volume of 25 µL containing: 12.5 µL deionized water, 2.5uL 5x buffer green, 2.5 µL 25mM MgCl, 1 µL nucleotide mixture, 2 µL primer (10 pmol/ µL), 4 µL template DNA. Parameters for PCR were as follow: 1 cycle of 2 min at 94 ºC for *A. niger* isolates, 45 cycles for *A. flavus* and 35 cycles for *A. niger* aggregate of 1 min at 92 oC (denaturation), 1 min at 35 oC annealing, 2 min at 72 oC (extension) and finally 1 cycle of 5 min at 72 oC. The amplified DNA were separated by electrophoresis in 2 % (w/v) agarose (Bio-Rad, California, USA) in 10X TBE buffer and stained with ethidium bromide solution (10mg/mL) (Promega, Madison, WI, USA) and visualized under UV light. The molecular masses of amplified DNA were estimated by comparing with a 1 Kb plus DNA ladder (Thermo Scientific, California, USA).

RESULTS

Specific primers were used to amplify specific regions to confirm morphological identification of the isolates. In all, a total of four *A. niger*, seven *A. flavus* and three *A. fumigatus* isolates were tested for amplification using each primer pairs ITS1/NIG and ITS1/FLA respectively. Single fragments estimated to be 400-500 bp amplified was detected only for suspected *A. flavus* when ITS1/FLA was tested (Figure 1).

Figure 1. Gel electrophoresis of PCR products of *Aspergillus* sp. amplified by using TS1/FLA; Lanes 1, 5, 6, 8, 9, 10 & 11: *A. flavus isolates*; Lanes 2, 3, 4 & 7: *A. niger* isolates Lanes 12, 13, 14: *A. fumigatus* isolates and Lanes; M: DNA marker

Isolates that did not amplify previously were suspected to be either *A. niger* isolates or *A. fumigatus*, which when further amplified using ITS1/NIG also showed single bands of between 400-500 bp (Figure 2) and were identified as *A. niger.*

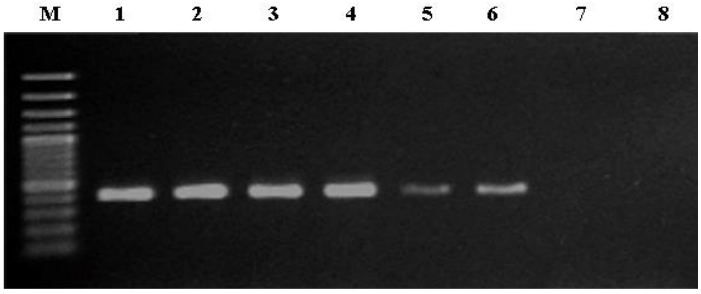

Figure 2. *PCR amplification profile of genomic DNA of two isolates of Aspergillus species* using ITS1/NIG; Lanes 1-6: *A. niger* isolates; Lanes 7, 8: *A. fumigatus*; Lanes M: DNA marker

Isolates that failed to amplify using ITS1/FLA and ITS1/NIG were confirmed as *A.fumigatus* and showed no amplification of the genomic DNA for *A. fumigatus* (Figure 1 and 2).

Genotypic Studies

Random Amplification Polymorphic DNA (RAPD) analyses were conducted using 8 primers (OPC-01, OPC-02, OPC-03, OPC-04, OPC-05, OPC-06, OPC-07, and OPC-08) for detecting similarity through amplified polymorphism between the genomic DNA of isolated *A. niger* and *A. flavus* strains and their respective control. For *A. niger* isolates, similar banding patterns between bands 4 to 8 denoting random amplification of polymorphic DNA were detectable with primers OPC-02, OPC-03, OPC-04, OPC-05, OPC-06, and OPC-08 (Figure 3.) being the most interpretable. Primers OPC-01 and OPC-07 produced amplification that resulted into faint polymorphic bands. All isolates identified as *A. niger* showed identical sequence pattern that are also identical to banding in *A. niger* control samples. Primer OPC-03 and OPC-05 displayed the faintest yet the highest discriminatory power (7) and OPC- 01 and OPC-06 the least (4).

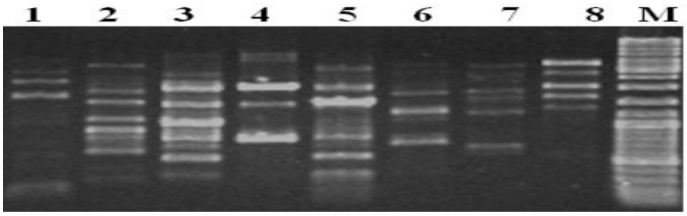

Figure 3. RAPD patterns of *A. niger* isolate, generated with OPC primers: Lane 1: OPC-01, Lane 2: OPC-02, Lane 3: OPC-03; Lane 4: OPC-4; Lane 5: OPC-5; Lane 6: OPC-06; Lane 7: OPC-07; Lane 8: OPC-08; Lane M: DNA marker

Similarly, *A. flavus* isolates also demonstrated similar banding with OPC primers with the highest (8) being with primer OPC 01 and the least (4) with OPC 02. Relatively more distinct bands were observed when primers OPC were used in amplification with genomic DNA of *A. flavus* isolates compare to *A. niger*. Except for primer OPC-05, all primers used produced distinct and interpretable polymorphic bands (Figure 4).

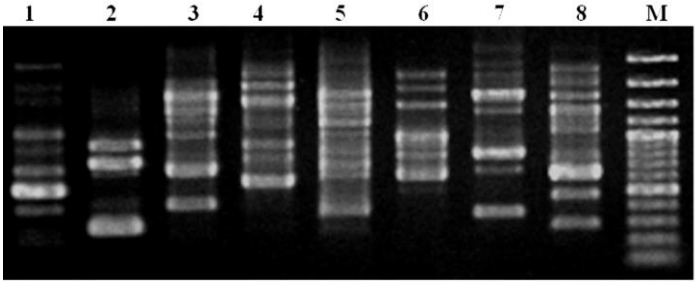

Figure 4. RAPD banding patterns of *A. flavus* isolate, generated with OPC primers: Lane 1: OPC-01, Lane 2: OPC-02, Lane 3: OPC-03; Lane 4: OPC-4; Lane 5: OPC-5; Lane 6: OPC-06; Lane 7: OPC-07; Lane 8: OPC-08; Lane M: DNA marker

All isolates identified as *A. niger* showed identical sequence pattern that are also identical to banding in *A. niger* control samples, similar polymorphic banding was also observed between isolates and *A. flavus* control when primer OPC-03 was amplified with genomic DNA from *A. flavus* isolates (Figure 5).

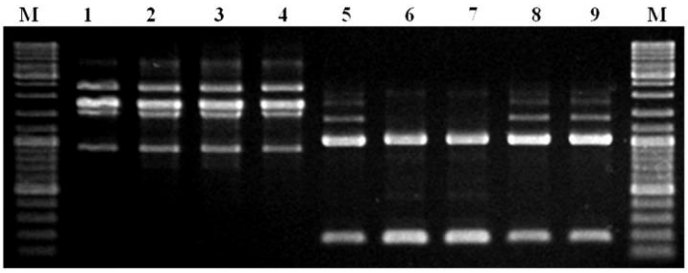

Figure 5. RAPD banding patterns of *A. niger* and *A. flavus* isolates and their respective controls, generated with primer OPC-03: Lane 1: *A. niger* control; Lanes 2-4: *A. niger* isolates; Lane 5: *A. flavus* control; Lanes 6-9: *A. flavus* isolates; M: DNA marker

DISCUSSION

The presence of Aspergillus sp. implies a risk of mycotoxin production and represents a health risk for the consumers [13]. According to Pittet [14], the mycotoxins produced by *Aspergillus* sp. include aflatoxins and ochratoxin A (OTA). *Aspergilus niger* and *Aspergilus flavus* are considered primary producers and contaminants of Ochratoxin A (OTA), and Aflatoxin (AF) respectively that are mycotoxin with lethal effects including immune suppressive, teratogenic and carcinogenic consequences, in groundnut and its associated products. *A. flavus* and *A. niger* have been the course of AF and OTA production in groundnut and its by-products [15, 16]. This results also agree with the study of Bernice and Jianmei [16] using ap-PCR and phylogenic analysis based on ITS and IGS sequences for *Aspergillus flavus*. The data obtained are also consistent with several other studies [17, 18]. Therefore, a close relationship appeared to exist between the isolates and the control, confirming the identification of potential ochratoxin and Aflatoxin producing *A. niger*, and *A. flavus* in studied groundnut products in Sokoto State, Nigeria.

Molecular characterization also facilitated proper identification as *Aspergillus* Sp. of three isolates which had been previously described as *A. flavus*. *A. niger* and A. *fumigatus* on the basis of morphological parameters. This result reinforces the fact that *A. flavus* has uncommonly been found as a contaminant in crops such as groundnut kernel [19]. Species identification based on morphological and biochemical characters is time consuming and not always precise; however, genetic similarity between species of *Aspergillus flavus*, as well as a high degree of intraspecific variability, also can prevent a clear differentiation of various species by molecular means. In the last decade, development of molecular methods for distinction of aflatoxigenic *A. flavus* and *A. niger* has been focused on aflatoxin biosynthesis genes.

Random amplified polymorphic DNA (RAPD) fingerprints were used to analyse genetic relationships among *A. niger*, and A. *flavus*. RAPD markers used to gain rapid and precise information about genetic similarities and dissimilarities of different Aspergillus species. RAPD fingerprints of *A. niger* and *A. flavus* revealed polymorphism in the analyzed *Aspergillus* sp. [19]. RAPD study is useful in estimating distances between and within same *Aspergillus* species and might help future programs of management and conservation. Genetic differences between species of the same genus maintain characterization and genetic diversity within this population [16]

CONCLUSION

Genotypically, clear evidence of the presence of potential AF and OTA producing *A. flavus* and *A. niger* isolates respectively was established through molecular PCR amplification of ITS regions and close RAPD-PCR similarity matrix found between the isolates and their respective references. The findings of this research suggested that Groundnut products could be contaminated with high levels mycotoxigenic fungi (*A. niger and A.flavus*), which is an indication factor for the real and confirmed presence of Aflatoxin, ochratoxin A and other mycotoxins making several products of groundnut samples unsafe for human consumption and as animal feeds ingredients for feed formulation.

There is therefore need for reducing the pathogenic fungi by treatment of seed for obtaining the good quality of seed and also reduce the mould fungi and mycotoxins production by improving the storage conditions and processing techniques

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