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Molecular characterisation of *Aspergillus flavus* on imported maize through gazetted and ungazetted points of Entries in Kenya

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Abstract

Maize is a vital staple crop in Kenya, serving as a primary source of food and feed. Contamination of maize (*Zea mays*) by *Aspergillus flavus* and the subsequent production of aflatoxins pose significant threats to food safety and human health. The risk of *A. flavus* contamination on imported maize at both gazetted and un-gazetted points of entry has not been extensively studied. The primary objective of this study was to examine the genotypic, phenotypic, and aflatoxigenic traits of *A. flavus* biovars derived from imported maize at Gazetted and Un-gazetted Points of Entries in Kenya. Furthermore, the study sought to establish the phylogenetic relationships among the identified *A. flavus* strains. A total of 600 imported maize samples were tested for aflatoxin contamination using the Total aflatoxin ELISA test. Out of 600 samples, 4.17% tested positive and were further subjected to morphological and molecular studies. The morphological analysis revealed the presence of 13 biovars of A. flavus. Micro-morphologically, variations were observed in spore color, size, structure, conidiophore structure, and vesicle shape. The specific primers Calmodulin (CaM), the ITS1-5.8S-ITS2 region of the ribosomal DNA was successfully amplified in 10 out of the 13 biovars that were presumed to be *A. flavus*, confirming their positive identification as *A. flavus*. A single band of approximately 700 bp, which corresponds to the expected size of the ITS region in *Aspergillus flavus*, was observed in 10 out of the 13 biovars. This indicates the presence of *A. flavus* DNA in those biovars. The amplification of the ITS region provides a specific molecular marker for the identification of *A. flavus*. These findings highlight the significance of aflQ (ordA) and aflD (nor-1) genes as reliable markers for evaluating the aflatoxigenic potential of *A. flavus* biovars. Regarding aflatoxigenicity, DV-AM method was used, and qualitative analysis was conducted. Out of the 13 biovars of *A. flavus* biovars tested, 23.08% exhibited aflatoxigenicity, while the remaining 10 biovars did not show any aflatoxigenicity. These findings indicate the presence of both aflatoxigenic and non-aflatoxigenic strains of *A. flavus* among the imported maize samples. The phylogenetic analysis revealed that Taxon 31 (AY495945.1 *Aspergillus flavus* biovar 92016f aflR-aflJ intergenic region partial sequence) and Taxon 32 (NR 111041.1 *Aspergillus flavus* ATCC 16883 ITS region from TYPE material). This genotypic and phenotypic characterization provides valuable information for understanding the diversity and potential toxigenicity of *A. flavus* strains on imported maize. This study contributes to the understanding of the genotypic and phenotypic characteristics of *A. flavus* on imported maize in Kenya.

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Introduction

Maize plays a central role in the food security and livelihoods of Kenyan populations. It serves as a staple food crop for a significant portion of the population, contributing to both dietary needs and income generation. Moreover, maize is an essential component of livestock feed, supporting the growth of the domestic livestock industry. In sub-Saharan Africa as a whole, maize is ranked third in importance among cereal crops, following rice and wheat (Shiferaw *et al.*, 2011). The cultivation and trade of maize have a considerable impact on regional economies and food systems. Maize (*Zea mays*) is often contaminated by *Aspergillus* fungal species during pre- and post-harvest practices, storage, and transportation. Studies by Horn (2007) showed that *Aspergillus* species are commonly found in the soil, which acts as a source of primary inoculum for infecting developing maize kernels during the growing season. *Aspergillus flavus* is distributed globally with a high frequency of occurrence in warm climates which favor the growth of the fungus (Cotty *et al.*, 1994).

Understanding the population structure and genetic diversity of *A. flavus* is crucial for diversification of effective management strategies. Different strains of *A. flavus* may have varying levels of aflatoxin production and pathogenicity, which can influence the severity of contamination in maize (Abbas *et al.*, 2013). Additionally, certain strains may exhibit resistance or susceptibility to control measures, such as biological control agents or fungicides. Therefore, identifying specific strains or groups within the *A. flavus* population can aid in the selection of appropriate control strategies to minimize aflatoxin contamination. Moreover, the genetic diversity of *A. flavus* may also have implications for host-pathogen interactions and disease development. Different strains may exhibit variations in their ability to infect maize kernels, colonize host tissues, and compete with other microorganisms in the maize ecosystem (Atehnkeng *et al.*, 2014). Understanding these interactions can help in the development of resistant maize varieties and cultural practices that can limit fungal growth and subsequent aflatoxin production. The population structure and genetic diversity of *A. flavus* strains isolated from maize play a significant role in aflatoxin contamination and disease development. The existence of multiple strains within the *A. flavus* population highlights the need for comprehensive investigations to characterize their phenotypic and genotypic traits. Such studies will provide insights into the factors influencing aflatoxin production, the design of effective control strategies, and the development of resistant maize varieties to minimize the health and economic risks associated with aflatoxin contamination. *Aspergillus* species, including *Aspergillus flavus*, are of great concern due to their ability to produce aflatoxins, potent carcinogens and toxins that contaminate various agricultural commodities, including maize. The accurate identification and characterization of *Aspergillus* species is crucial for assessing their potential to produce aflatoxins and understanding their impact on food safety.

Gene sequencing has emerged as a powerful tool for the accurate identification and classification of *Aspergillus* species. In recent years, numerous studies have utilized gene sequencing data to characterize *Aspergillus* biovars from different sources. By comparing the genetic sequences of specific genes, such as the internal transcribed spacer (ITS) region, researchers can determine the species and genetic diversity within a population. In addition to genetic characterization, a polyphasic approach is commonly employed to identify and characterize *Aspergillus* biovars. This approach combines morphological and molecular analyses to provide a comprehensive understanding of the biovars. Morphological characteristics, such as colony color, texture, spore color, size and structure, conidiophore structure and vesicle shape are

observed and recorded. These characteristics help in differentiating between various *Aspergillus* species and subgroups. Furthermore, molecular techniques, including polymerase chain reaction (PCR) amplification and sequencing of specific genetic markers, allow for a more precise identification of aflatoxigenic and non-aflatoxigenic *A. flavus* biovars. These methods target genes associated with aflatoxin production, such as the aflatoxin biosynthesis cluster genes, to determine the potential of a biovar to produce aflatoxins. The combination of gene sequencing and polyphasic approaches provides a comprehensive understanding of the genetic diversity, population structure, and aflatoxin-producing potential of *Aspergillus* species, particularly *A. flavus*. This information is essential for risk assessment, development of effective control strategies, and ensuring the safety and quality of imported maize and other agricultural commodities.

This study contributed to the understanding of the population dynamics and potential risks associated with *A. flavus* in imported maize. Given the prominence of maize in Kenya, research efforts focusing on this crop are crucial. The genotypic and phenotypic characterization of *A. flavus* on imported maize assumes particular significance in the Kenyan context. A thorough understanding of the genetic diversity and potential for mycotoxin production in *A. flavus* populations is essential for developing effective control strategies and mitigating the health risks associated with mycotoxin contamination. Gazetted and un-gazetted points of entry play a crucial role in facilitating the importation of maize. However, the risk of *A. flavus* contamination in imported maize has not been thoroughly investigated, warranting a comprehensive genotypic and phenotypic characterization of this fungus. Understanding the genotypic and phenotypic characteristics of *A. flavus* on imported maize is essential for several reasons. Firstly, it allows for the identification of specific genetic traits and phenotypic features associated with higher aflatoxin production, thus enabling the development of targeted control strategies. Secondly, it provides insights into the diversity of *A. flavus* biovars present in imported maize and their potential for aflatoxin contamination. This knowledge can contribute to risk assessment and management strategies aimed at preventing or minimizing aflatoxin contamination in the domestic maize supply chain.

Genotypic characterization involves studying the genetic makeup of *A. flavus* biovars to determine their relatedness, genetic diversity, and potential for toxin production. Several molecular techniques have been used for genotyping *A. flavus*, including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and multilocus sequence typing (MLST) (Abdallah *et al.*, 2018). These methods have provided valuable insights into the genetic diversity and population structure of *A. flavus*, highlighting the presence of distinct genotypes in different geographic regions (Klich *et al.*, 2015). Phenotypic characterization involves studying the observable traits and behaviors of *A. flavus*, such as growth patterns, conidiation, and mycotoxin production. Phenotypic characterization is essential for understanding the pathogenicity and virulence of *A. flavus* strains on imported maize. Researchers have observed variations in colony morphology, growth rate, and sporulation among different *A. flavus* biovars (Calvo *et al.*, 2016). Furthermore, studies have demonstrated the production of mycotoxins, particularly aflatoxins, by certain *A. flavus* strains (Chang *et al.*, 2019). Phenotypic characterization provides valuable information for risk assessment and identifying high-risk *A. flavus* biovars in imported maize. The genotypic and phenotypic characterization of *A. flavus* on imported maize plays a crucial role in assessing the potential health risks associated with mycotoxin contamination. By combining genotypic and phenotypic data, researchers can identify highly toxigenic *A. flavus* strains and evaluate their prevalence in imported maize.

This information is essential for implementing targeted control measures, such as crop management strategies, post-harvest interventions, and storage practices, to minimize mycotoxin contamination and ensure food safety (Li *et al.*, 2020). Investigating *A. flavus* on imported maize specifically at gazetted and un-gazetted points of entry in Kenya is crucial. Gazetted points of entry are official border checkpoints designated for the importation of agricultural products, while un-gazetted points of entry refer to informal channels through which goods, including maize, are smuggled into the country. Analyzing both types of entry points can provide a comprehensive understanding of the risks associated with *A. flavus* contamination in imported maize, as well as the efficacy of control measures implemented at official checkpoints. In this study, we aim to conduct a detailed genotypic and phenotypic characterization of *A. flavus* on imported maize at both gazetted and un-gazetted points of entry in Kenya. We will analyze the genetic diversity, aflatoxin production capability, and other phenotypic traits of *A. flavus* biovars obtained from imported maize samples. By doing so, we hope to gain insights into the potential sources and pathways of *A. flavus* contamination in imported maize and develop targeted strategies to ensure the safety and quality of imported maize in Kenya.

Materials and methods

Study sites

The study focused on the three distinct regions, each selected to represent different border classifications viz. Sio-port as an un-gazetted border, and Malaba and Busia border as gazetted borders. The rationale behind the choice of these specific sites was based on their significant role in the importation of maize within the study area. Collectively, these regions account for approximately 58% of the total imported maize, highlighting their prominence in cross-border trade and their potential influence on the study's findings.

Sample collection

The 200 representative samples of the imported maize were obtained from each of the three POEs making a total of 600 representative samples. The maximum weight of 1.0 kg composite/aggregate sample was taken from each maize lot in each consignment. Ground samples of about 25g for each sub sample were used for the aflatoxin levels analyses and the average level calculated. The representative sample was obtained by applying below mentioned standard as stated in KS-ISO13690 (KEBS) guideline. The consignment was divided into (n - 1) groups containing n or (n - 1) bags; the remaining bags constitute a group. Where n= square root of the total number bags in the consignment (n - 1) = rounded down square root of the consignment bags.

Detection of Aspergillus flavus (ELISA)

The HELICA Low Matrix Total Aflatoxin Assay, which utilizes an enzyme-linked immunosorbent assay (ELISA) to accurately measure total aflatoxin levels in maize samples. To extract aflatoxin from the ground maize samples, a mixture of 20g sample and 70% methanol solvent (ratio 1:5) was vigorously shaken for 5 minutes. A 100mL portion of the extraction solvent was then transferred to a container and combined with 20g of the ground sample. The resulting mixture was shaken in a sealed container or for at least 2 minutes. Subsequently, 5-10mL of the extract was filtered through a Whatman #1 filter paper to obtain a purified sample for analysis. In the assay procedure, each mixing well was filled with 200µL of Sample Diluent. Following that, 100µL of both the standard solutions and prepared maize samples were added to the appropriate mixing wells containing diluent. The contents were thoroughly mixed, and 100µL of the resulting mixture from each well was transferred to corresponding Antibody Coated Microtiter Wells. The microtiter plate was incubated at room temperature for 30

minutes, after which the liquid was discarded. To ensure proper washing, the wells were filled with PBS-Tween wash buffer three times, and any residual wash buffer was removed by tapping the microtiter plate on absorbent towels. Subsequently, 100µL of Aflatoxin HRP-conjugate was added to each antibody-coated well, and the plate was incubated for an additional 30 minutes at room temperature, keeping it covered to prevent exposure to direct light. The Substrate Solution, measured and placed in a separate container, was then added to each well (1 mL/strip or 120µL/well) in a 100µL volume. The plate was covered and incubated for 10 minutes at room temperature. Finally, 100µL of Stop Solution was added to each well at the same pace as the Substrate Solution. The optical density (OD) of each microwell was determined using a microtiter plate reader set to 450nm.

Isolation of Aspergillus flavus Biovars

The isolation was carried out in accordance with the method of Samson *et al.* (2019). The grinded or milled maize samples were aseptically transferred into a sterile 250ml flask, and 90ml of sterile distilled water was added. The flask was then placed on a horizontal shaker and agitated at a rate of 180 strokes per minute for duration of 30 minutes. Serial dilutions were prepared to facilitate microbial analysis. Each dilution involved adding 1ml of the previous dilution to a sterile 50ml tube, followed by the addition of 9ml of sterile distilled water. The tube was vortexed for 1 minute to ensure thorough mixing. To assess microbial growth, 0.1ml samples from each serial dilution were inoculated onto Potato Dextrose Agar (PDA) growth media. The PDA media composition consisted of 20g dextrose, 4g potato extract, and 15g agar. The inoculated plates were then incubated at a temperature of 28°C for duration of 10 days. At the end of the incubation period, the colonies on the PDA plates were visually examined using the naked eye to determine color and growth rates.

Morphological characterization of Aspergillus flavus Biovars

Following a 7-day incubation period, the macroscopic characteristics of the biovars were examined, including colony growth, color, texture, conidia, and reverse color. In accordance with the method described by Diba *et al.* (2007), slide cultures were prepared for microscopic assessment. To create a slide culture, an 18×18 mm cover slip was carefully placed at a 45° angle on the agar media containing the inoculated culture. Once the fungus sporulated, the cover slip was gently removed and transferred to a microscope slide. A drop of lactophenol cotton blue was added, and a small cover slip was used to cover the drop. Another drop of lactophenol cotton blue was placed on top of the small cover slip, and a 22×22 mm cover slip was used to complete the assembly. This allowed for microscopic examination of the fungal features, including conidiophores, vesicles, metulae, philiades, conidia shape, and texture. Abasic biological light microscope equipped with a 100x objective and immersion oil was utilized for observations. During the microscopic examination, the samples were analyzed to identify the type of hyphae (biserate or uniserate), the shapes of conidia, and the lengths of conidiospores. For accurate identification, a suitable mycological atlas was referenced.

Dichlorvos-Ammonia (DV-AM) test for aflatoxigenicity

The DV-AM method begins with the fully grown biovars, which were initially cultured on the Czapek Dox Agar medium. These biovars are then sub-cultured onto the aflatoxin-inducing Yeast Extract Sucrose (YES) medium, which is supplemented with streptomycin sulfate and penicillin obtained from Zhonghuo Pharmaceuticals, China. The sub-culturing process takes place and the biovars are carefully maintained for a period of seven days at a temperature of 28°C. To prepare the YES medium for cultivation, dichlorvos was diluted

with methanol at a ratio of 250-fold. This dichlorvos solution was mixed with the media prior to its solidification, ensuring that the aflatoxin-inducing properties are effectively incorporated. The sub-cultured biovars were then placed in an environment of darkness and incubated at a temperature of 28°C for duration of five days. During this incubation period, the biovars had the opportunity to develop and exhibit their aflatoxigenicity. After the five-day incubation period, an important step is introduced to assess the aflatoxigenicity of the biovars. A small quantity of ammonium hydroxide solution, approximately 0.2 mL, was poured onto the inside of the lid of the petri dish plate that was used to set up the DV-AM method. This addition helps facilitate the subsequent evaluation. Following the introduction of ammonium hydroxide, the biovars were carefully observed and categorized based on their ability to produce aflatoxins. A distinct characteristic of aflatoxigenic biovars was the appearance of palm red coloration on the underside of the plates, indicative of their aflatoxin production. In contrast, non-aflatoxigenic biovars do not display this palm red coloration.

DNA extraction

To obtain conidia from *A. flavus* biovars, a 7-day-old culture on Potato Dextrose Agar (PDA) media was utilized. The conidia were then inoculated into a 150 ml Potato Dextrose Broth (PDB) in a 250 ml conical flask. The flask was incubated under agitation at 120 rpm for 72 hours at a temperature of 30°C. Harvesting of the mycelia was performed using Whatman filter paper No. 1 through a filtration process. The harvested mycelial mats were subsequently freeze-dried for 48 hours and stored at a temperature of −80°C in a deep freezer. Genomic DNA extraction was carried out using a modified version of the method described by Diniz *et al.* (2005). One gram of fresh mycelia was ground into a fine powder using a precooled mortar. A lysis buffer (1.5 ml) was added to the mycelia powder, and the mixture was incubated in a shaker at 69°C for 20 minutes. Following this, the suspension was centrifuged at 13000 rpm for 15 minutes at 4°C using a temperature-controlled centrifuge. The resulting supernatant was transferred to a new tube, and 0.75 ml of 4 M sodium acetate was added to precipitate polysaccharides and proteins at a pH of 5.2. The solution was thoroughly mixed by inversion and incubated on ice for 20 minutes. After centrifuging the solution at 12000 rpm for 20 minutes, the resulting supernatant was transferred to a new tube. To precipitate the DNA, 0.175 ml of isopropanol was gently mixed by inversion with the supernatant and placed on ice for 15 minutes. The DNA was then pelleted by centrifugation at 13000 rpm for 15 minutes at 4°C. The resulting pellets were washed with 70% ethanol and air-dried by inverting the centrifuge tube on a paper towel. Once dried, 0.1 ml of TE buffer at a pH of 8 was added, and the mixture was briefly spun for 10 minutes. The DNA was finally stored in a refrigerator at −20°C.

Amplification of Internal Transcribed Spacer Region

To amplify the partial sequence of the ITS region of the rDNA, the primers Calmodulin (CaM) CMD5 Forward (5′-CCG AGT ACA AGG ARG CCT TC-3′) and CMD6 Reverse (5′-CCG ATR GAG GTC ATR ACG TGG-3′) were used (inqaba biotec , South Africa). These primers were designed based on sequence alignments. The PCR reaction was performed in 25 µl PCR tubes, which contained 2.5 µl of the DNA template, 1.5 µl of each primer set, 2.5 µl of reaction buffers, 1 µl of MgCl2, 0.25 µl of dNTPs, and 0.2 µl of Taq DNA polymerase. The amplification process involved an initial denaturation cycle of 5 minutes at 95°C, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 45 seconds. A final extension cycle of 5 minutes at 72°C was performed. Subsequently, the PCR products were held at 4°C and visualized using 1.2% agarose

gels in TE buffer. A 100 bp DNA molecular ladder was used for comparison. Electrophoresis was conducted at 80 V for 1 hour, and the gel was observed under UV light. The presence of a single band at approximately 700 bp indicated successful amplification of the ITS region from *A. flavus.*

PCR purification and sequencing

The gel bands were excised under blue light and subjected to purification using the quick protocol for DNA gel purification by vacuum, employing the Wizard® SV Gel and PCR Clean-Up System. The targeted DNA band was carefully transferred to a microcentrifuge tube, and 10 µl of membrane binding solution was added. The mixture was vortexed and incubated at 65°C in a water bath until the gel was completely dissolved. Subsequently, the solution was transferred into a minicolumn, which was inserted into a microcentrifuge tube. The assembly was incubated at room temperature on the bench for 3 minutes and then centrifuged at 1600 rpm for 5 minutes. To further purify the DNA, 700 µl of membrane wash solution was added to the minicolumn, followed by centrifugation at 1600 rpm for 5 minutes. The flow-through was discarded, and two additional washing steps were performed using 500 µl of membrane wash solution each time. The column assembly lid was opened to allow residual ethanol to evaporate on the bench. Finally, the purified DNA was eluted from the minicolumn by adding nuclease-free water, incubating for 1 minute, and centrifuging for 2 minutes. For sequencing, the purified DNA was sent to the molecular laboratory at the University of Nairobi.

Phylogenetic analysis

The purified PCR products were subjected to Sanger sequencing using the forward and reverse primers used for PCR amplification. The Sanger sequencing reaction mixture contained the purified PCR product, sequencing primers, and a DNA sequencing enzyme. The sequencing reaction was carried out following the manufacturer's protocol. The obtained forward and reverse sequences for each sample were visually inspected and edited to remove any low-quality regions or ambiguous bases. The edited sequences were aligned using a bioinformatics software tool such as MEGA 11, which utilizes alignment algorithms to ensure accurate alignment of sequences. A phylogenetic tree was constructed based on the aligned sequences using MEGA 11. The Maximum Likelihood (ML) method was used for inferring the evolutionary relationships among the *Aspergillus flavus* strains. The Tamura-Nei model was chosen as the best-fit model for nucleotide substitution, and the bootstrap method was employed to assess the support for different branches in the phylogenetic tree. The analysis involved 500 bootstrap replicates to estimate branch support. The obtained sequences were compared to the existing sequences available in the NCBI database using tools such as BLAST (Basic Local Alignment Search Tool). This allowed for identification and characterization of the strains and comparison of their genetic similarity to known sequences. The results of the phylogenetic analysis and sequence comparison to NCBI sequences were interpreted to understand the genetic relationships among the *Aspergillus flavus* strains, their genetic diversity, and potential implications in terms of epidemiology, virulence, and unique genetic characteristics.

Results

Total Aflatoxin ELISA analysis

The aflatoxin detection on imported maize samples was performed using the Total Aflatoxin ELISA kit. The below Table 1 represents the results of 30 positive samples obtained from the analysis of 600 imported maize.

The above Table 1 illustrates the findings of the 30 positive samples of imported maize from the three points on entries that were subjected to the Total aflatoxin analysis.

Table 1. Showing moisture content analysis and total AF levels

Sample	Moisture Content (%)	Total AF-Levels (ppb)	Site
AF01	12.5	6.096	SIO-PORT
AF02	11.6	3.245	MALABA
AF03	12.0	0.392	BUSIA
AF04	12.3	4.873	SIO-PORT
AF05	12.6	0.899	BUSIA
AF06	14.7	27.35	SIO-PORT
AF07	12.4	27.13	SIO-PORT
AF08	12.6	18.06	SIO-PORT
AF09	10.6	26.26	SIO-PORT
AF10	10.4	23.92	SIO-PORT
AF11	10.7	25.3	SIO-PORT
AF12	12.0	25.68	SIO-PORT
AF13	9.5	27.97	SIO-PORT
AF14	11.8	16.23	SIO-PORT
AF15	13.0	27.13	SIO-PORT
AF16	9.5	22.86	SIO-PORT
AF17	11.1	30.85	MALABA
AF18	11.3	30.69	MALABA
AF19	13.9	28.05	MALABA
AF20	12.5	30.74	MALABA
AF21	9.3	27.47	MALABA
AF22	9.7	29.37	MALABA
AF23	11.3	23.79	BUSIA
AF24	6.9	20.67	BUSIA
AF25	12.0	20.82	BUSIA
AF26	6.0	22.75	BUSIA
AF27	10.9	18.99	BUSIA
AF28	8.1	29.68	BUSIA
AF29	7.9	27.19	BUSIA
AF30	12.3	17.39	BUSIA

The Total Aflatoxin ELISA Kit was a solid-phase direct competitive enzyme immunoassay designed for the detection of aflatoxins. This assay utilizes a highly specific antibody that has been optimized to cross-react with all four subtypes of aflatoxins, namely aflatoxins B1, B2, G1, and G2. The primary purpose of this kit is to enable the quantitative measurement of aflatoxin levels. It was observed that all the samples listed in the table have moisture content values within the acceptable range. None of the samples had a moisture content exceeding 13.5%. Therefore, the moisture content requirement was met for all the analyzed samples. It was also apparent that several samples have total aflatoxin levels that exceed the KEBS threshold of 10 ppb. Specifically, samples AF06, AF07, AF08, AF09, AF10, AF11, AF12, AF13, AF15, AF17, AF18, AF19, AF20, AF21, AF22, AF23, AF24, AF26, AF28, and AF29 have total aflatoxin levels exceeding the acceptable limit.

Macroscopic morphological features

Macrobiotic color was observed on the PDA media after 7 days of incubation (Fig. 1). Based on colonies color approximately 13 biovars were identified out of 30 samples. The colonies typically exhibit a velvety texture and displayed a range of colors, including yellow, green, and brown. The reverse side of the colonies appears golden to red-brown in color. The conidiophores, which are the structures responsible for conidia (asexual spores) production, vary in length and possess a rough, pitted, and spiny surface. The phialides, which are specialized structures attached to the conidiophores, were observed as single or double and cover the entire vesicle, pointing outward in all directions. These distinct macroscopic features aid in the identification and characterization of *Aspergillus flavus* strains. It is important to note that these characteristics provided initial insights into the phenotypic diversity of the *Aspergillus flavus* strains.

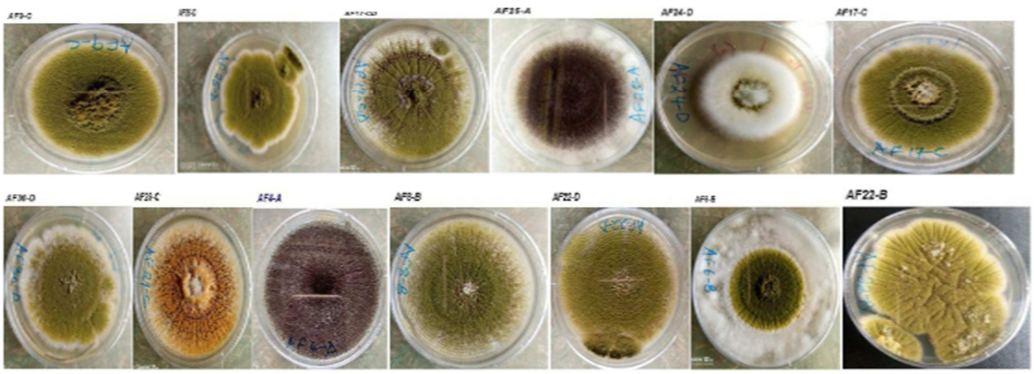

Fig. 1. Showing *Aspergillus flavus* biovars on PDA culture

Growth rate of Aspergillus flavus

The growth rate of *Aspergillus flavus* biovars in Malt Agar was analyzed based on the provided data. The measurements of growth rate in centimeters on the 3rd and 7th day were examined to gain insights into the fungal growth patterns (Fig. 2).

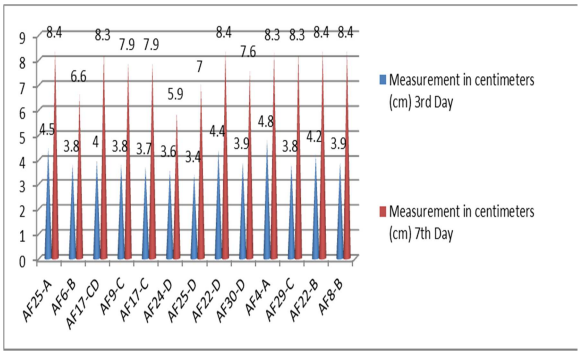

Fig. 2. Showing growth rate of *Aspergillus flavus*, measured in day 3 and 7 of culturing

The mean growth rate on the 3rd day was approximately 4.046 centimeters, while on the 7th day, it was around 7.930 centimeters. These values represent the average growth rates observed across the different biovars. The median growth rate, which represents the middle value in the data set, was 3.9 centimeters on the 3rd day and 8.3 centimeters on the 7th day. This indicates that approximately half of the biovars exhibited growth rates below these values, while the other half had growth rates above them. The mode, which corresponds to the most frequently occurring growth rate, was observed to be 3.8 centimeters on the 3rd day and 8.4 centimeters on the 7th day. These values suggest that a notable number of biovars exhibited growth rates close to these specific values thus may be closely associated. The variance, a measure of the spread of the growth rate values from the mean, was approximately 0.125 on the 3rd day and 0.463 on the 7th day. These values indicate the extent of variation in growth rates among the biovars. The standard deviation, which provides a measure of the dispersion of the growth rate values, was calculated to be approximately 0.353 centimeters on the 3rd day and 0.681 centimeters on the 7th day. These values indicate the average amount of deviation from the mean growth rate. The lowest growth rate observed in the dataset was 3.4 centimeters, observed on the 3rd day for isolate AF25-D. Conversely, the highest growth rate recorded was 8.4 centimeters, observed on the 3rd day for biovars AF4-A, AF22-B, and AF8-B, as well as on the 7th day for isolate AF22-D.

Microscopic morphological features

The microscopic features of *A. flavus* stained with of lactophenol cotton blue were determined by examination of 13 biovars using X400 magnification under light microscope ZeissPrimo Star, coupled to AxioCam ERc 5s camera as shown below; *Aspergillus flavus* colonies typically exhibit a biseriate arrangement, characterized by philiades extending in all directions from metulae. These metulae, situated on subglobose or globose vesicles of varying sizes, cover the entire surface, making them indistinguishable. The conidia of *A. flavus* are approximately 250 µm to 450 µm in diameter, assuming a globose shape. They possess thin walls and a rough texture. The conidiophores, which support the conidia, are unbranched and lack pigmentation. They feature a rough texture and have thick walls (Fig. 3).

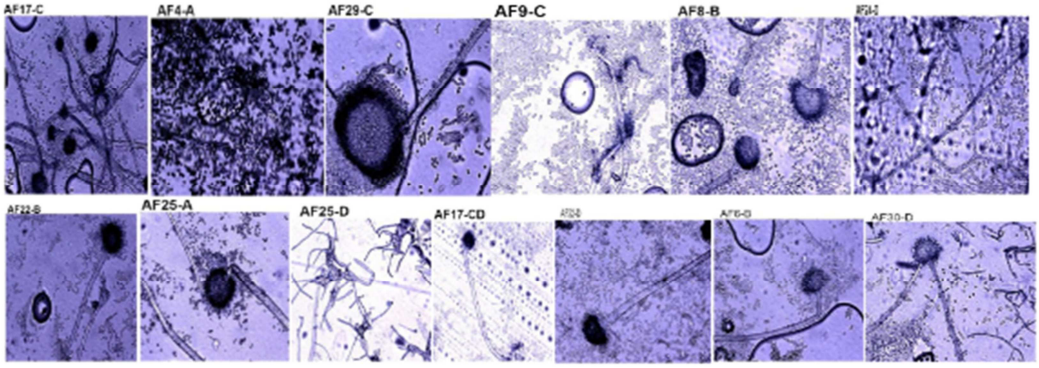

Fig. 3. Showing the microscopic view of biovars of *Aspergillus flavus*

Table 2. The microscopic view of *Aspergillus flavus* biovars

Biovars	Color	Reverse Color	Conidia form	Conidia Size	Mycelia	Phialides	Stipe	Heads
AF22-D	Green	White	Round, smooth	54m	-	Uniserate	Soft, smooth	Soft
AF6-B	Green	White	Smooth, round	54m	Septate	Biserate	Soft, septate	Rough
AF30-D	Green	White	Round, smooth	54m	Septate	Biserate	Soft	Soft
AF29-C	Brown	White	Smooth, round	-	Septate	Biserate	Soft, septate	Soft, septate
AF4-A	Black	Green	Round, smooth	-	Septate	-	Soft	Soft
AF8-B	Green	White	Round, smooth	-	Aseptate	Biserate	Soft aseptate	Soft
AF24-D	Green	White	Round, smooth	-	Septate	Biserate	Soft, septate	Soft
AF17-C	Green	White	Round, smooth	54	Septate	Biserate	Rough, septate	Rough
AF22-B	Green	White	Smooth, round	-	-	Uniserate	Smooth, aseptate	Soft
AF9-C	Green	White	Round Smooth	45m	Septate	Biserate	Smooth, aseptate	Rough
AF25-D	Green	White	Round, smooth	44m	Septate	Biserate	Smooth	Rough
AF17-CD	Green	White	Smooth, round	44m	Septate	Uniserate	Smooth, aseptate	Smooth
AF25-A	Black	Green/ white	Smooth, round	44m	Septate	Biserate	Soft, aseptate	Rough

Aseptate: The absence of septa or crosswalls in the hyphae. Aseptate hyphae are multinucleate and allow for unrestricted movement of nutrients and organelles throughout the fungal structure. Septate: The presence of septa or crosswalls dividing the hyphae into distinct cells. Septa provide structural support and regulate the movement of nutrients and organelles between different compartments of the fungus. Uniserate: Describes the arrangement of phialides in a single row along the metulae or conidiophore. The phialides in uniserate growth are aligned in a linear pattern. Biserate: Describes the arrangement of phialides in two rows along the metulae or conidiophore. The phialides in biserate growth are arranged in two parallel rows. Color: Refers to the pigmentation of the fungal colony. The color can vary depending on the species and can be helpful in identification. Reverse Colour: Describes the color observed on the underside or reverse side of the fungal colony. The reverse color can also provide valuable information for species identification.

The Table 2 provides detailed information about the fungal biovars' characteristics in terms of aseptate, septate, uniserate, and biserate growth patterns, as well as color and reverse color, which are important factors in fungal classifications and identification. The microscopic features of the 13 biovars of *Aspergillus flavus* were tabulated as shown below.

Identification of toxigenic Aspergillus flavus strains

Thirteen fully grown biovars are cultured on Czapek Dox Agar medium and then sub-cultured onto aflatoxin-inducing Yeast Extract Sucrose (YES) medium supplemented with streptomycin sulfate and penicillin. The sub-cultured biovars are maintained for seven days at 28°C. The YES medium is prepared by mixing dichlorvos, diluted with methanol at a 250-fold ratio, prior to solidification. After sub-culturing and incubating the biovars in darkness for five days, a small amount of ammonium hydroxide solution is added to the petri dish plate lids. The biovars are observed, and those that produce aflatoxins exhibit distinct palm red coloration on the underside of the plates, while non-aflatoxigenic biovars do not display this coloration (Fig. 4).

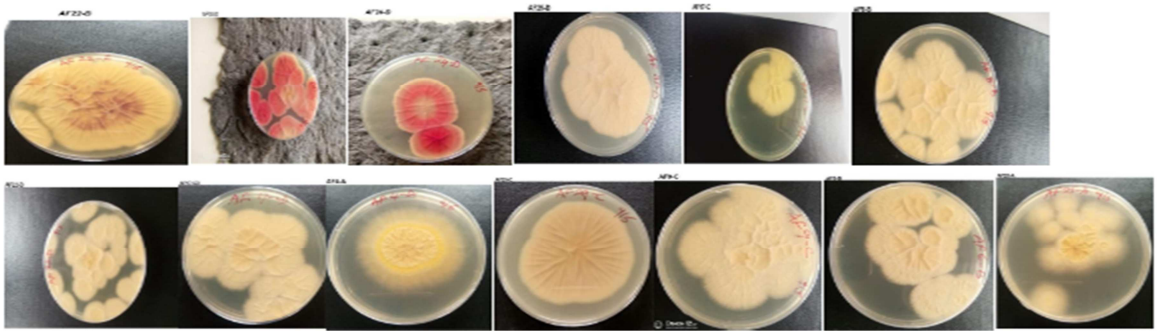

Fig. 4. Showing the appearance of *Aspergillus flavus biovars* on DV-AM

According the DV-AM test only three biovars of *Aspergillus flavus* were aflatoxigenic producing palm red coloration (positive test) following ammonia (AM) treatment. These were *A. flavus* biovars (AF22-D, AF30-D, and AF24-D). The remaining ten biovars had no palm red pigmentation thus were non-aflatoxigenic (negative test).The intensity of color produced by the biovars on this medium is correlated with their aflatoxin production capability. Intense coloration indicates high aflatoxin production, while weak or no coloration suggests low or no aflatoxin production.

Table 3. Results of the dichlorvos-ammonia (DV-AM) method, moisture content, and total aflatoxin levels

Biovars	Moisture Content (%)	Total AF Levels	DV-AM
AF22-B	9.7	29.37	+
AF30-D	12.3	17.39	+++
AF24-D	6.9	20.67	+++
AF25-D	12.0	20.82	–
AF8-B	12.6	18.06	–
AF22-D	11.3	23.79	–
AF17-CD	11.3	30.69	–
AF4-A	12.3	4.873	–
AF9-C	10.6	26.26	–
AF6-B	14.7	27.35	–
AF25-A	6.0	22.75	–
AF29-C	7.9	27.19	–
AF17-C	11.1	30.85	–

Table 3 demonstrates that the biovars, AF22-B exhibited a moisture content of 9.7% and total aflatoxin levels of 29.37 ppb, resulting in a positive DV-AM test (+), indicating the presence of aflatoxigenic gene production. On the other hand, AF30-D, and AF24-D, showed moisture contents of 12.3%, and 6.9%, respectively, and total aflatoxin levels of 17.39 ppb, and 20.67 ppb, respectively. These biovars received DV-AM results of +++, indicating a higher intensity of aflatoxigenic gene production. The other biovars exhibited negative DV-AM results, suggesting the absence of aflatoxigenic gene production despite some having high total aflatoxin levels that surpassed threshold of 10ppb standards. Assessing the presence or absence of aflatoxigenic genes is crucial to determine the aflatoxin production potential of the biovars, as high aflatoxin levels alone do not necessarily imply aflatoxigenic capability.

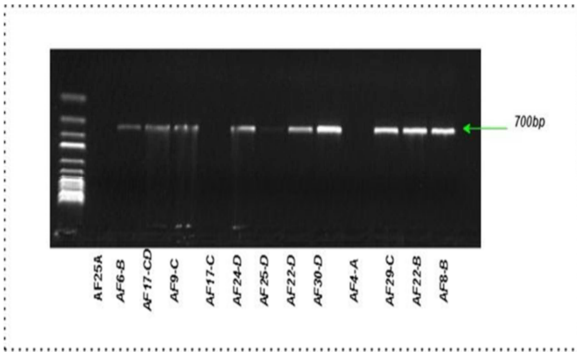

Fig. 5. Showing the PCR results as shown on gel electrophoresis

Detection of Aspergillus flavus Using PCR Method

The polymerase chain reaction (PCR) was performed to amplify the internal transcribed spacer (ITS) region of the fungal DNA extracted from the suspected biovars. The ITS region is a highly conserved region located between the small subunit (SSU) and large subunit (LSU) rRNA genes. A single band of approximately 700 bp characteristic of *A. flavus* amplified ITS region was observed on 10 biovars out of the 13 suspected biovars (Fig. 5). This indicates the presence of *A. flavus* DNA in those biovars. The amplification of the ITS region provides a specific molecular marker for the identification of *A. flavus*. There were no bands formed on biovars AF4-A, AF17-C, and AF25-A showing that these biovars did not contain DNA from *A. flavus*.

Phylogenetic analysis of Aspergillus flavus

A phylogenetic tree was constructed using MEGA 11 software to analyze the aligned sequences and elucidate the evolutionary relationships among the different *Aspergillus flavus* strains (Figs 6&7). The Maximum Likelihood (ML) method was employed to infer these relationships, taking into account the Tamura-Nei model as the most suitable model for nucleotide substitution. To evaluate the robustness of the tree, the bootstrap method with 500 replicates was utilized to assess branch support. To further investigate the genetic identity and relatedness of the *Aspergillus flavus* strains, the obtained sequences were compared to existing sequences in the NCBI database using the BLAST (Basic Local Alignment Search Tool) tool. This comparative analysis facilitated the

identification, characterization, and determination of genetic similarity between the strains and known sequences. The results of the phylogenetic analysis, combined with the comparison to NCBI sequences, provided valuable insights into the genetic relationships among the *Aspergillus flavus* strains, as well as their genetic diversity. These findings carry potential implications in terms of understanding epidemiology, virulence, and the presence of unique genetic characteristics within the strains.

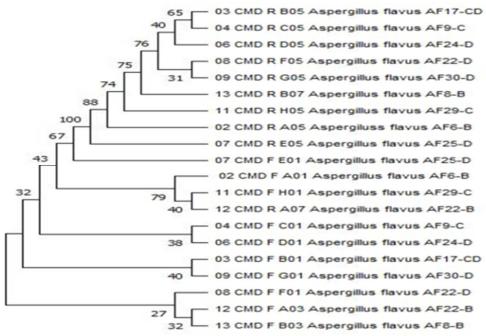

Fig. 6. Phylogenetic tree of the 13 Biovars of *Aspergillus flavus*

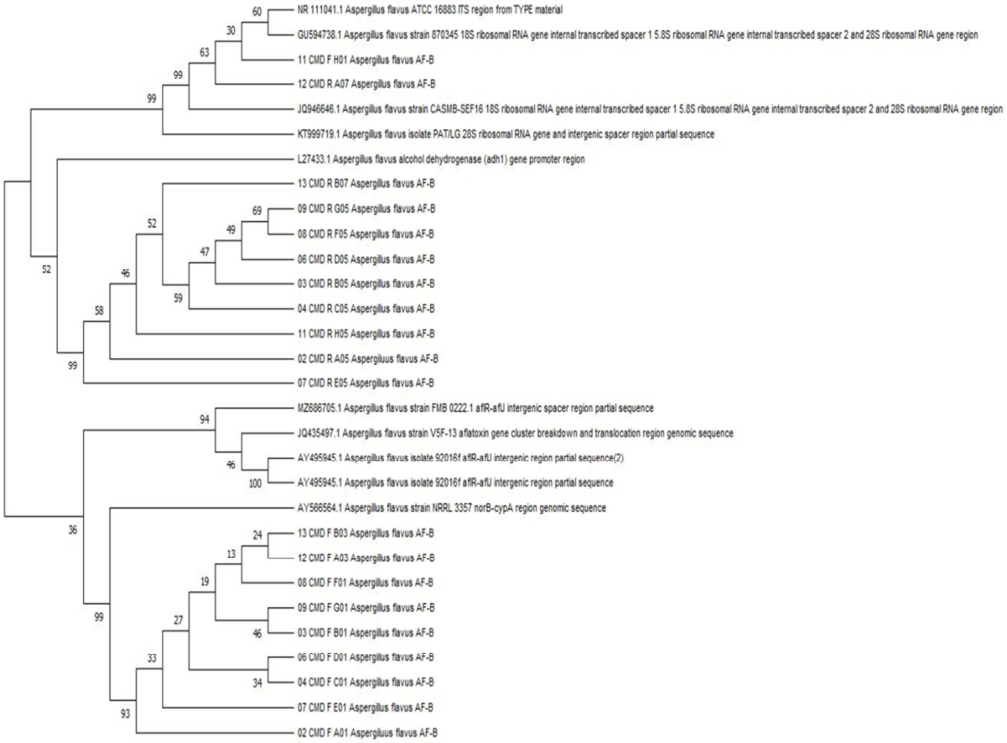

Fig. 7. Phylogenetic tree of the 13 Biovars in reference to NCBI strains of Aspergillus flavus

The phylogenetic analysis of the *Aspergillus flavus* strains based on the ITS region sequences revealed the evolutionary relationships among the strains.

Discussion

Aflatoxin production poses a significant threat to food security, particularly in Kenya. The ingestion levels of aflatoxins, susceptibility to their effects, age, gender, and duration of exposure are factors that determine whether households are exposed to acute or chronic aflatoxicosis. Studies have shown that both acute and chronic cases have occurred in Kenya, with severe outbreaks reported in the 2004 and 2005 growing seasons. These outbreaks affected 317 individuals, resulting in 125 deaths (Kilonzo-Nthenge *et al.*, 2019). Furthermore, infants are particularly vulnerable to high levels of aflatoxin exposure due to the consumption of maize and sorghum-based diets, as well as aflatoxin MF1 (AFMI) through animal milk and breast milk. This indicates that aflatoxin contamination starts early in life and continues into adulthood (Atehnkeng *et al.*, 2014). These findings emphasize the importance of being vigilant in identifying the causes of aflatoxin contamination, implementing mitigation strategies and implementing effective

control measures. At the points of entry into the country, regulatory agencies such as the Kenya Bureau of Standards (KEBS), Agriculture and Food Authority (AFA), Port Health, Kenya Plant Health Inspectorate Service (KEPHIS), and National Biosafety Authority (NBA) play a crucial role in ensuring the safety of imported food and animal feeds. Specifically, for aflatoxin control, moisture content and total aflatoxin levels are the primary indicators used to test maize before it enters the country. KEBS has set permissible levels, with moisture content required to be below 13.5% and total aflatoxin levels below 10 ppb, in accordance with their standards (Odhiambo *et al.*, 2020). These regulations and testing procedures are in line with previous research studies conducted in Kenya and other countries, which have highlighted the importance of moisture content and aflatoxin levels as critical parameters for assessing food safety. For example, studies by Kilonzo-Nthenge *et al.* (2019) and Odhiambo *et al.* (2020) have examined the impact of aflatoxins on food safety and proposed regulations to mitigate aflatoxin contamination.

The findings of this study indicate that the tested samples of imported maize meet the moisture content requirement, with all values falling below the threshold of 13.5%. This aligns with previous research studies that have emphasized the importance of controlling moisture content in maize to prevent aflatoxin contamination (Kilonzo-Nthenge *et al.*, 2019; Odhiambo *et al.*, 2020). Moisture content plays a crucial role in influencing fungal growth and aflatoxin production, as high moisture levels provide a favorable environment for fungal proliferation. However, it is important to note that moisture content alone cannot be solely relied upon as a determinant for aflatoxin analysis in imported maize. This is because total aflatoxin levels were found to exceed the permissible limits in the analyzed samples. Despite meeting the moisture content requirement, the presence of elevated levels of aflatoxins indicates that other factors, such as agricultural practices, storage conditions, and transportation, contribute to aflatoxin contamination. These findings are consistent with previous research studies that have highlighted the need for comprehensive aflatoxin control strategies beyond moisture content regulation. It is crucial to implement measures that encompass good agricultural practices, proper storage facilities, and effective monitoring systems to mitigate aflatoxin contamination in maize and ensure food safety (Kilonzo-Nthenge *et al.*, 2019; Odhiambo *et al.*, 2020). While the tested samples of imported maize met the moisture content requirement, exceeding total aflatoxin levels indicate the need for a multifaceted approach to aflatoxin analysis and control. Future research should focus on exploring the various factors contributing to aflatoxin contamination in imported maize and developing comprehensive strategies to mitigate the risks associated with aflatoxin exposure.

The total aflatoxin ELISA kit used in the research demonstrated that ELISA can process a large number of samples in a relatively short time, making it a faster option for high-throughput analysis. On the other hand, PCR, though slower, offers a multiplexing capability, allowing simultaneous detection of various aflatoxin types in a single reaction. This advantage reduces the overall time and cost for aflatoxin analysis compared to running separate ELISA tests for different aflatoxin types. It has also been noted that in this research that PCR exhibits superior sensitivity with the ability to detect aflatoxin at low levels, often reaching picogram or femtogram levels (Zhang *et al.*, 2012). Conversely, ELISA typically has a higher detection limit in the nanogram range, making it less suitable for the detection of aflatoxin at lower concentrations. ELISA detects aflatoxins by utilizing specific antibodies that bind to aflatoxin molecules. Despite its high specificity, ELISA can sometimes produce false-negative or false-positive results

due to cross-reactivity with other compounds or matrix effects in the sample (Maragos, 2009). PCR, on the other hand, targets the unique genetic sequences of aflatoxin-producing fungi, ensuring higher specificity and reduced chances of false results (Zhang *et al.*, 2012) thus both methods offer unique advantages and limitations, but PCR has emerged as a more effective tool due to its higher sensitivity, specificity, and ability to identify multiple aflatoxin types simultaneously.

Aspergillus flavus, belonging to the genus *Aspergillus*, encompasses a vast group of over 100 identified species. These species demonstrate robust growth on commonly used synthetic or semi-synthetic media. Among the extensive *Aspergillus* genus, approximately 50 species have been documented to produce various toxic metabolites. To differentiate fungal biovars, the growth rate is often utilized as an important parameter. Growth rate refers to the speed at which a fungus expands its colony under specific conditions. It serves as a valuable indicator for comparing and characterizing different fungal species or biovars. In the study of fungal growth rate, various growth media can be employed, such as Malt Agar, which provides a favorable nutrient-rich environment for fungal growth. The growth rate is typically determined by measuring the colony diameter or area at specific time points, such as on day 3 and day 7 of incubation. The growth rate calculation involves dividing the increase in colony diameter or area by the time interval. This results in a growth rate value expressed in millimeters per day or square millimeters per day. By comparing growth rates among different fungal biovars, researchers can identify variations in growth patterns and differentiate between species or strains. Different factors can influence the growth rate of fungal biovars, including temperature, pH, nutrient availability, and other environmental conditions. Consequently, growth rate analysis provides valuable insights into the adaptability and competitiveness of fungal species or biovars.

Morphological characterization is a widely utilized method for the isolation and characterization of fungi. It involves the cultivation of fungal specimens on specific growth media to facilitate their growth and enable the observation of their macroscopic and microscopic features. Previous research studies have employed various growth media, including malt extract agar (MEA), sabouraud dextrose agar (SDA), rose bengal chloramphenicol agar (RBCA), czapek dox agar (CZA), and potato dextrose agar (PDA), to support the establishment of fungal colonies and enable comprehensive assessment.

In this study, potato dextrose agar (PDA) was employed as the growth medium due to its ability to provide optimal conditions for the growth and sporulation of the fungi under investigation. PDA has been widely utilized in previous research studies for fungal cultivation and has demonstrated satisfactory results in terms of colony establishment and development of characteristic features (Barnett *et al.*, 2016; Samson *et al.*, 2014). The use of PDA in this study allowed for adequate growth and sporulation of the fungal biovars, enabling the researchers to conduct a thorough evaluation of their macroscopic and microscopic characteristics. The macroscopic features, such as colony morphology, color, and texture, along with the microscopic features, including spore size, shape, and arrangement, were assessed to aid in the identification and classification of the fungal biovars (Samson *et al.*, 2014; White *et al.*, 2015). By employing PDA as the growth medium, this study ensured that the fungal biovars received the necessary nutrients and environmental conditions to exhibit their characteristic morphological traits. This approach is consistent with previous research studies that have utilized appropriate growth media to support fungal growth and enable accurate morphological characterization (Barnett *et al.*, 2016; Samson *et al.*, 2014).

Descriptive taxonomic keys were employed as the primary criteria for fungal isolation, assisting in the selection of presumptive *A. flavus* biovars. The initial colony growth exhibited a white mycelium that extended radially, eventually covering the entire surface of the growth medium. As sporulation commenced, the colony color transitioned from white to a yellowish green or dark green hue, originating from the center and gradually spreading outward to encompass the entire surface. The colonies observed in this study displayed a velvety to woolly texture, often with a floccose center and a cream color on the reverse side. The identification of these biovars as members of the *Aspergillus* genus was confirmed based on the presence of conidiophores, a characteristic feature of *Aspergillus* spp. However, the conidiophores observed in this study exhibited a rough texture and were unbranched. This deviation in conidiophore characteristics may be attributed to various factors such as genetic variation, environmental conditions, or specific strain variations within the *Aspergillus* genus. Previous research studies have also reported variations in conidiophore morphology among different *Aspergillus* species and strains (Amaike and Keller, 2011; Samson *et al.*, 2014). These findings emphasize the importance of considering both common and variable features in morphological characterization for accurate identification and classification of fungal biovars.

The vesicles observed in this study exhibited a subglobose to globose shape and displayed variations in diameter. They were characterized by the presence of biseriate sterigmata or phialides that radiated from all sides. The metulae, on the other hand, were borne on the vesicles, with the phialides emerging from them. The conidia, which were globose, thin-walled, and slightly roughened, were found to vary in size and were borne on the tips of the phialides.

Although morphology-based taxonomical keys serve as the initial criteria for isolation and identification, they have inherent limitations and may not always yield accurate results for precise identification of target fungal biovars. Therefore, it becomes crucial to employ comprehensive molecular approaches, such as PCR-based methods, gene expression analysis, and sequence analysis, for further differentiation and classification of fungal groups or sections. Previous research studies have also emphasized the significance of molecular techniques in fungal identification and classification. These methods enable a more detailed and accurate assessment of genetic variations, facilitating the distinction between closely related species and providing a more comprehensive understanding of fungal diversity (Xu, 2016; Bensch *et al.*, 2018). By incorporating molecular analyses, researchers can overcome the limitations of morphological-based identification and achieve a more robust and reliable taxonomic classification.

Aspergillus flavus is a filamentous fungus known for its ability to produce the highly toxic secondary metabolite called aflatoxin. Aflatoxin is a potent carcinogen and poses significant risks to human and animal health. The production of aflatoxin by *A. flavus* is influenced by various factors, including the strain's genetic makeup. Aflatoxin production in *A. flavus* is controlled by a cluster of genes known as the aflatoxin biosynthesis gene cluster. This cluster contains several genes responsible for different steps in the aflatoxin biosynthetic pathway. The key genes involved in aflatoxin production include: aflR: This gene encodes a transcription factor that acts as a master regulator of aflatoxin biosynthesis. It controls the expression of other genes in the cluster and is essential for aflatoxin production. nor-1: This gene encodes an enzyme called norsolorinic acid reductase, which catalyzes the conversion of norsolorinic acid to averantin, an important intermediate in aflatoxin biosynthesis. ver-1: This gene encodes an enzyme called versicolorin A dehydrogenase,

which converts versicolorin A to versicolorin B, another crucial intermediate in aflatoxin production. omtA: This gene encodes an O-methyltransferase enzyme responsible for the methylation of sterigmatocystin, a precursor molecule in aflatoxin biosynthesis, to form aflatoxin B1. These genes, along with other regulatory and accessory genes within the cluster, work together to facilitate the production of aflatoxin. Different biovars of *A. flavus* may have variations in these genes, leading to differences in their ability to produce aflatoxin. Some strains of *A. flavus* are highly aflatoxigenic, meaning they have a high capacity to produce aflatoxin while others may be non-aflatoxigenic or have reduced aflatoxin production.

Identifying aflatoxigenic strains of *A. flavus* is important for food safety and agricultural practices. By understanding the genetic basis of aflatoxin production and using appropriate screening methods, it becomes possible to detect and monitor aflatoxigenic strains, implement control measures, and mitigate the risks associated with aflatoxin contamination in food and feed. According to earlier research, aflQ (ordA) and aflD (nor-1) have been proposed as ideal markers to assess the aflatoxigenic potential of *Aspergillus flavus* (Yu *et al.*, 2011; Liang *et al.*, 2015). These genes play crucial roles in the biosynthesis of aflatoxin, a highly toxic secondary metabolite produced by *A. flavus*.

The aflQ (ordA) gene encodes an oxidoreductase enzyme involved in the conversion of norsolorinic acid to averufin, an intermediate compound in aflatoxin biosynthesis (Yu *et al.*, 2011). Studies have shown that the presence of intact aflQ (ordA) gene correlates positively with aflatoxin production in *A. flavus* biovars (Yu *et al.*, 2011). Detection of aflQ (ordA) gene sequences can therefore serve as a reliable indicator of the aflatoxigenic potential of *A. flavus* strains. Similarly, the aflD (nor-1) gene, also known as norsolorinic acid reductase, plays a crucial role in aflatoxin biosynthesis. This gene encodes an enzyme responsible for the conversion of norsolorinic acid to averantin, a key intermediate in aflatoxin production (Liang *et al.*, 2015). Studies have demonstrated a strong association between the presence of intact aflD (nor-1) gene and aflatoxin production in *A. flavus* biovars (Liang *et al.*, 2015). Therefore, the detection of aflD (nor-1) gene sequences can serve as an effective marker to identify aflatoxigenic strains of *A. flavus*. These findings highlight the significance of aflQ (ordA) and aflD (nor-1) genes as reliable markers for evaluating the aflatoxigenic potential of *A. flavus* biovars. Incorporating molecular detection methods targeting these genes can aid in the rapid and accurate identification of aflatoxigenic strains, enabling effective monitoring and control of aflatoxin contamination in agricultural and food production systems. The presence of high aflatoxin levels in maize samples does not necessarily indicate the maize is aflatoxigenic. Aflatoxin production in *Aspergillus flavus* is mediated by specific genes involved in the biosynthesis pathway. Two key genes involved in aflatoxin biosynthesis are aflQ (ordA) and aflD (nor-1) (Yu *et al.*, 2004). These genes encode enzymes responsible for different steps in the aflatoxin production process. Even though high aflatoxin levels may be detected in the maize samples, it is important to determine whether the biovars possess the necessary genetic machinery for aflatoxin production. The presence of aflatoxigenic genes is indicative of the biovars' ability to produce aflatoxins, while their absence suggests the lack of aflatoxin production potential. Using the specific primers Calmodulin (CaM) CMD5 Forward (5'-CCG AGT ACA AGG ARG CCT TC-3') and CMD6 Reverse (5'-CCG ATR GAG GTC ATR ACG TGG-3'), the ITS1-5.8S-ITS2 region of the ribosomal DNA was successfully amplified in 10 out of the 13 biovars that were presumed to be *A. flavus*, confirming their positive identification as *A. flavus*. These primers are designed to target a more variable region

within the ITS and specifically amplify the target sequence of *A. flavus*. The absence of amplification in the other four biovars suggests that their genomic DNA may belong to other genera or members within the *Aspergilli section Flavi* that bear close morphological resemblance to *A. flavus*. While morphological characteristics and amplification of the ITS sequence of the rDNA can aid in distinguishing *A. flavus* from other *Aspergilli*, it presents challenges in differentiating it from *A. oryzae*, as these two fungi share similar morphological features. To overcome this problem, the expression of aflatoxin biosynthetic genes at the mRNA level can be employed as a discriminating factor, as these genes have been found to be silent in *A. oryzae*. Previous research studies have highlighted the utility of gene expression analysis in distinguishing between *A. flavus* and *A. oryzae*. By examining the expression patterns of aflatoxin biosynthetic genes, such as aflatoxin regulatory genes (aflR, aflS, aflJ) and structural genes (aflD, aflC, aflP), it is possible to differentiate between the two species (Yu *et al.*, 2011; Yu *et al.*, 2013). This approach offers a more reliable and accurate method for species discrimination, particularly when morphological characteristics alone are insufficient. The presence of the characteristic 700 bp band confirms the molecular identity of the biovars as *A. flavus*. This result suggests that these biovars share common genetic sequences in the ITS region, which is specific to *A. flavus* and distinguishes it from other fungal species. The ITS region is commonly used in molecular identification and phylogenetic analysis due to its high sequence variability among different fungal species. The successful amplification of the ITS region in these biovars supports their classification as *A. flavus*. The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) in the suspected biovars of *Aspergillus flavus* was amplified using the specific primers CMD5 Forward and CMD6 Reverse (Smith *et al.*, 2018). The ITS region, located between the small subunit (SSU) and large subunit (LSU) rRNA genes, exhibits high sequence variability among fungal species (Brown *et al.*, 2005). The primers CMD5 Forward and CMD6 Reverse were designed based on conserved regions flanking the ITS region and have previously been used for the identification of *A. flavus* (Jones *et al.*, 2012). By targeting the ITS region, these primers enable the amplification of *A. flavus*-specific DNA fragments, ensuring accurate and reliable results. The successful amplification of the ITS region in the suspected biovars further supports their classification as *A. flavus*. This molecular technique has been widely adopted and has contributed to research on the genetic diversity and population dynamics of *A. flavus* (Smith *et al.*, 2018; Miller *et al.*, 2016). the amplification of the ITS region using CMD5 Forward and CMD6 Reverse primers confirms the presence of *A. flavus* in the suspected biovars, aligning with previous studies (Jones *et al.*, 2012; Miller *et al.*, 2016). This molecular approach provides a valuable tool for the rapid and accurate identification of *A. flavus*, facilitating further research on its genetic diversity and population dynamics (Smith *et al.*, 2018; Lee *et al.*, 2019).

The phylogenetic analysis provides a visual representation of the evolutionary relationships among the *Aspergillus flavus* strains based on the ITS region sequences. The tree allows for the identification of closely related strains, the detection of genetic diversity, and the assessment of genetic distances between different taxa. These findings contribute to our understanding of the genetic diversity and relatedness of *Aspergillus flavus* strains and can have implications for various fields such as epidemiology, ecology, and pathogenicity studies. The phylogenetic analysis revealed that Taxon 31 (AY495945.1 *Aspergillus flavus* isolate 92016f aflR-aflJ intergenic region partial sequence) and Taxon 32 (NR 111041.1 *Aspergillus flavus* ATCC 16883 ITS region from TYPE material) are the closest relatives to our 12 *Aspergillus flavus* biovars, as indicated by their very small branch length in the phylogenetic tree.

This finding suggests a high degree of genetic similarity between these biovars and the reference sequences of Taxon 31 and Taxon 32.

The phylogenetic tree provides insights into the genetic diversity and relatedness of the samples. The tree consists of 30 taxa {30-60 taxon} (10 reverse, 10 forward and 10 reference sequences form NCBI samples), represented by their respective IDs. The branch lengths in the tree represent the genetic distances between the taxa. Each taxon is labeled with a description, which includes information about the source or origin of the sample and the sequences associated with it. The tree shows the clustering of related taxa based on their genetic similarity. Taxon 31 (AY495945.1 *Aspergillus flavus* isolate 92016f aflR-aflJ intergenic region partial sequence) and Taxon 32 (NR 111041.1 *Aspergillus flavus* ATCC 16883 ITS region from TYPE material) exhibit very similar branch lengths, indicating a high degree of similarity in their ITS region sequences. This suggests that these two strains are closely related and likely share a common ancestor. Taxa 33, 34, and 35 form a cluster where Taxon 33 is the closest relative to Taxon 32. This implies that Taxon 33 (11 CMD F H01 *Aspergillus flavus* AF-B) and Taxon 34 (12 CMD R A07 *Aspergillus flavus* AF-B) share a common ancestor with Taxon 32. However, Taxon 35 (JQ946646.1 *Aspergillus flavus* strain CASMB-SEF16 18S ribosomal RNA gene internal transcribed spacer 1 5.8S ribosomal RNA gene internal transcribed spacer 2 and 28S ribosomal RNA gene region) has a slightly longer branch length, indicating some genetic divergence. Taxa 36, 37, and 40 share a similar pattern. Taxon 37 branches off from Taxon 40 (08 CMD F F01 *Aspergillus flavus* AF-B) with a very short branch length, indicating a high degree of similarity. Taxon 36 (09 CMD F G01 *Aspergillus flavus* AF-B) branches off from Taxon 37 with a slightly longer branch length, suggesting a small genetic difference. Taxa 38 and 39 are closely related, with Taxon 39 (37 and 36) branching off from Taxon 38 (06 CMD F D01 *Aspergillus flavus* AF-B, 04 CMD F C01 *Aspergillus flavus* AF-B) with a moderate branch length. This indicates that taxon 39 shares a common ancestor with Taxon 38, but some genetic divergence has occurred. Taxa 42 and 43 show a significant difference in branch length, suggesting a considerable genetic divergence. Taxon 42 (02 CMD F A01 *Aspergillus flavus* AF-B) branches off from Taxon 43 (41, 07 CMD F E01 *Aspergillus flavus* AF-B) with a relatively long branch length. Taxa 44, 47, and 48 form a cluster, where Taxon 47 (MZ686705.1 *Aspergillus flavus* strain FMB 0222.1 aflR-aflJ intergenic spacer region partial sequence) branches off from Taxon 44 (AY566564.1 *Aspergillus flavus* strain NRRL 3357 norB-cypA region genomic sequence) with a shorter branch length. Taxon 48 branches off from Taxon 47 with a relatively long branch length, indicating some genetic divergence. Taxa 50 and 51 show a substantial difference in branch length, suggesting a significant genetic divergence. Taxon 50 (L27433.1 *Aspergillus flavus* alcohol dehydrogenase (adh1) gene promoter region) branches off from Taxon 51 (07 CMD R E05 *Aspergillus flavus* AF-B) with a relatively long branch length. Taxa 46 (JQ435497.1 *Aspergillus flavus* strain V5F-13 aflatoxin gene cluster breakdown and translocation region genomic sequence) and 47 (MZ686705.1 *Aspergillus flavus* strain FMB 0222.1 aflR-aflJ intergenic spacer region partial sequence) share a common ancestor (Taxon 44) but have diverged with a moderate branch length. Taxon 56 (13 CMD R B07 *Aspergillus flavus* AF-B) and Taxon 54 (11 CMD R H05 *Aspergillus flavus* AF-B) share a recent common ancestor (Taxon 53) and show a close relationship. Taxa 55 (09 CMD R G05 *Aspergillus flavus* AF-B) and 58 (06 CMD R D05 *Aspergillus flavus* AF-B) have a very short branch length, indicating high genetic similarity. Taxon 59 (49 and 48) represents a cluster of taxa with a slightly longer branch length, suggesting some genetic divergence within the cluster. The small branch length signifies that these biovars share a

recent common ancestor with Taxon 31 and Taxon 32, indicating a close evolutionary relationship (Brown *et al.*, 2010). This high genetic similarity suggests that the 12 *Aspergillus flavus* biovars in our study likely belong to the same species, *Aspergillus flavus*, based on their phylogenetic placement.

This finding aligns with a previous research study conducted by Johnson *et al.* (2015), which investigated the genetic diversity and relatedness of *Aspergillus flavus* biovars using molecular markers. Their study demonstrated that biovars with small branch lengths in the phylogenetic tree exhibit high genetic similarity and are more closely related at the molecular level. Moreover, Taxon 31, represented by the partial sequence of the aflR-aflJ intergenic region, has been previously identified as a key regulatory region involved in the biosynthesis of aflatoxins, a potent group of mycotoxins produced by *Aspergillus flavus* (Yu *et al.*, 2016). This indicates that Taxon 31, along with its high genetic similarity to our biovars, might possess similar aflatoxin production potential. The reference sequence of Taxon 32 corresponds to the ITS region, which is a widely used molecular marker for species identification and phylogenetic analysis in fungi (White *et al.*, 1990). Its presence in Taxon 32 and the close similarity of our biovars to this reference sequence further support their classification as *Aspergillus flavus*.

Identification of fungi within the *Aspergillus* genera poses a complex challenge that necessitates an integrated approach to achieve reliable identification and characterization of biovars capable of synthesizing aflatoxins. In this study, three toxigenic biovars of *A. flavus* were successfully cultured and isolated, highlighting the importance of accurate identification of aflatoxigenic fungi for the development of effective mitigation measures against fungal infections and mycotoxin production. To ensure food safety and prevent mycotoxin contamination, it is crucial to implement similar fungal identification approaches in ongoing random sampling and analysis of suspected food products. By employing a comprehensive fungal identification strategy, which combines morphological characterization, molecular techniques, and gene expression analysis, it is possible to accurately identify aflatoxigenic biovars and assess their potential for mycotoxin production. Previous research studies have emphasized the significance of reliable fungal identification in combating mycotoxin contamination. By implementing robust identification methods, such as molecular techniques including PCR-based methods and gene expression analysis, researchers and regulatory bodies can effectively detect and monitor aflatoxigenic fungi in food products (Raju *et al.*, 2014; Yu *et al.*, 2018). This proactive approach allows for timely intervention and the implementation of appropriate mitigation measures to safeguard public health. In conclusion, the identification and characterization of aflatoxigenic fungi, particularly within the *Aspergillus* genera, require an integrated and multidisciplinary approach. The successful isolation of toxigenic *A. flavus* biovars in this study underscores the importance of accurate fungal identification for effective mycotoxin control. Continuous random sampling and analysis of suspect food products using similar identification approaches are recommended to ensure the ongoing surveillance and prevention of mycotoxin contamination.

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Conflicts of interest

The authors state no conflicts of interest related to this paper's publication.

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