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Effect of Ultraviolet Radiation on the Phenotypic and Molecular Mutation in *Aspergillus flavus*

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ABSTRACT

The present study aimed to investigate the effect of ultraviolet radiation induced genetic mutations (phenotypic and molecular) in *Aspergillus flavus* collected from corn grains and tested for their ability to produce aflatoxin using the high liquid chromatography technique (HPLC). A Hanau Fluotest Forte 5261 UV test lamp was used for treating *A. flavus* at a transition wavelength of 254 nm for 24 h. The results showed that UV radiation exposure at a transition wavelength of 800 has a mutagenic effect on *A. flavus* since a change in the shape and acceleration of growth was observed compared to the non-exposed sample. In addition, the second-generation conidia showed slower growth than the non-exposed sample. The aflatoxin types AfB1, AfB2, AfG1, and AfG2 differed in quality and quantity based on fungal isolates. The concentrations ranged from 35.210 to 195.75 ug /kg of grains. It was found that *A. flavus* (UV) produced AfB1 at 35.250 ug/kg of seeds. Meanwhile, *A. flavus* (O) produced AFB1, AFB2, AFG1, and AFG2 with 45.74, 10.4, 15.24, and 135.20 ug/kg of grains, respectively. On the other hand, the genetic variability among the two isolates was detected. The DNA genome of *A. flavus* isolates (UV and O) was tested using PCR to amplify the FIR gene. PCR amplification confirms its inter-specificity to *A. flavus*, UV, and O isolates with expected sizes 1335 and 1346bp, respectively. Translating the lR gene nucleate sequence to amino acids revealed genetic variability among the two isolates of 445 and 448, respectively. The percentage of nucleotide sequence replacement was 0.89%, while the percentage for amino acid sequence replacement was 1.57%.

KEYWORDS: *Aspergillus flavus*, Mutation, Aflatoxin, HPLC, Corn grains

INTRODUCTION

Ultraviolet (UV)-irradiation, a type of ionized radiation, cleans and eliminates microscopic neighborhoods at wavelengths between 210-3800 nm.It is used to sterilize hospitals and eliminate different types of bacteria and fungi and toxin-producing *Aspergillus* sp. private, *Fusarium* sp., and *Botrytis* sp. types of mushrooms (Amit et al., 2003). The X-rays are of short wavelengths and have high permeability in living matter, causing aggregation of protein material within the pathogen, which leads to the rapid killing of these neighborhoods.

Recent studies have concluded that the missing food ratio because of fungus reached 30-25% or more, which represents a real threat to public health and a burden on the national economy. However, the use of irradiation technology (X-rays) increased the level of national production by eliminating 95% of the microbes without impacting the environment. Some states succeeded in conserving food using radiation; nearly two hundred thousand tons of rice, wheat, and barley were treated with X-rays, placed in high-specification stores, and used for human consumption without any registered symptoms. The irradiation process is quick but causes damage to humans and animals. Without raising the temperature, the DNA within the cells can be damaged. At specific doses, mutations can occur (Suttle 2004) due to damage to the nucleic acids and proteins (Caasi-Lit et al., 1997). An induced mutation can occur because of the strong absorption at different wavelengths, especially between 600-890 nm, causing photochemical dimerization acidosis to adjacent nucleic acids and damaging a series of DNA (Vreugdenhil 2004). UV irradiation can cause damage to skin cells and DNA molecules, resulting in abnormal covalent bonds between cytosine bases and productive bilateral units. Therefore, the DNA polymerase will read the bilateral unit as "AA." It will not read the original "CC," ultimately causing structural changes to the DNA, adding thymine during transcription, and distorting the shape of the DNA spiral. This also hinders the repair process, preventing integration; consequently, a mutation may appear. This study aims to induce and evaluate genetic mutations (phenotypic and molecular) of *Aspergillus flavus* using ultraviolet radiation.

MATERIALS AND METHODS

Isolation of *Aspergillus flavus*

According to the standard methodology described below, the potato dextrose agar medium (PDA) was used to isolate *A. flavus* from corn grains. One hundred micrograms per mL of streptomycin sulfate was used as an antibiotic to prevent the growth of undesirable bacteria. The corn grains were sterilized with 1% sodium hypochlorite solution for 2 min, washed with distilled water several times, and dried between two layers of sterile filter paper. Five grains were inoculated in a Petri dish containing PDA medium and incubated for 4 days at 25± 1ºC. The petri dishes were investigated to screen for a single colony of *A. flavus*. The colonies showed different colors and morphological characters; hence, the colony was sub-cultured separately on agar media until a pure culture was obtained by hyphal tip or the signal spore techniques. Each pure fungus was cultured on a PDA slant and used for further identification.

Identification of *Aspergillus flavus*

The slant of purified fungi was examined first by the naked eye at different magnifications of the stereomicroscope. A colony of fungus can be easily distinguished when viewed under high magnification. To identify *A. flavus* a specific Czapek agar media was used. The colony characteristics and the morphological features were recorded. The morphological structure of *A. flavus* depends on the head, stripe, vesicle, medullae, and conidia. The identification was carried out at the Plant Pathology Department, National Research Center, Cairo, Egypt, according to Maren and John (1983) and Mathur and Olga (2003).

Ultraviolet treatment

A. flavus collected from corn grains were tested under UV radiation using a Hanau Fluotest Forte 5261 UV test lamp for treatment at a transition wavelength of 254 nm for 24 h. Under sterilization conditions, a small amount of growth mycelium was collected by a needle and transferred to small glass tubes (10 mL) containing 5 mL of sterile distilled water. The tubes were exposed to the palm source radiation facing UV for 30 min. After

exposure, the fungus was transferred to PDA dishes and incubated at a temperature of 25 ± 1 C at a rate of four occurrences of check-fits. The morphological structure for *A. flavus* was detected using a microscope equipped with a camera. Samples were transferred to a glass slide containing lacto phenol and imaged using the supplied Blamer Date virtual form of the light microscope.

Determination of aflatoxins

Maize grains were artificially infected with two isolates of *A. flavus* after being moistened with 18% sterile water. UV is indicating treated isolates and O original, then incubated at 25°C for 15 days for aflatoxin production (Filtenborg et al., 1983).

Preparation of standard solution aflatoxins

Diluted portions of stock solution (0.5 µg/mL) in solvent solution benzene: acetonitrile (98:2, v/v) were used to prepare concentrations of aflatoxin standards B1, B2, G1, and G2. The standards' purity criteria were determined using chromatographic purity and molar absorption. The absorbance close to 350 nm was determined, and concentration was calculated, although it was not necessary if standards were obtained from a recognized source (Hustchins and Hagler 1983).

Extraction of aflatoxins

Fifty grams of corn grains infected with two isolates of *A. flavus* were blended. The powder was transferred into a 500 ml flask, and 25 ml of water, 25 g of diatomaceous earth (DE) and 250 ml of chloroform were added to the mixture. The flask was tightly covered u masking tape and shaken for 30 min to extract the toxins. The contents were then transferred to a Buchner funnel pre-coated with a layer of (DE) about 5 mm thick and filtered using a light vacuum in case of slow filtration. The first 50 mL of filtrate was collected for further analysis.

Determination of aflatoxins quantity by High-Performance Liquid Chromatography (HPLC)

Preparation of column chromatography

A ball of glass wool was loosely placed in the bottom of a 22 x 300 mm chromatographic column, and 5 grams of sodium sulfate anhydrous was added to the silica gel. The glass column was filled with ca. 40-50 ml of chloroform, and 10 g of activated silica gel was added. Then, a 15 g of sodium sulphate anhydrous was added to the surface of the column. Finally, 50 ml of the sample extract was applied to the column. One hundred fifty ml of n-hexane was used for de-fating, followed by 150 mL diethyl ether for de-pigmentation at a 5 mL/min flow rate. One hundred fifty ml of chloroform: methanol (97:3) was used to eluate the aflatoxins from the column at a 5 mL/min flow rate. The elution was concentrated using a rotary evaporator to ca. 1 ml and directly transferred to a vial. Finally, the solvent was evaporated and re-dissolved in a known volume of chloroform (0.2-1.0 ml) and kept in a vial for quantification (Shephard et al., 1990).

Derivatization

The derivatives of positive samples and standards were done as described in (AOAC 2000)

HPLC Conditions

The HPLC instrument (U.S.A) used for aflatoxins determination was a waters (474) system equipped with a quaternary pump fluorescence detector set system at 360 nm excitation and 440 nm emission wavelengths. Data were collected and integrated using Totalchrom Navigator Chromatography Manager Software (AOAC 2000).

Isolation of total DNA

The pellets were grown in Czapek broth medium followed by 5 days at 25°C with shaking at 200 rpm. After incubation, the mycelium mass was harvested by centrifugation at 6000 rpm for 10 min. The pellets were washed twice in 135 ml of buffer solution (145 Mm NaCl; 100 mM Na_2HPO_4; pH 7.5). Total genomic DNA was isolated using Chang et al.'s method (1995).

Amplification of aflR gene DNA

The aflR gene got amplified by PCR using a DNA Thermostable polymerase (COT thermo cycler model 1105). The aflR gene was amplified using primer sets (table-1) which were designed for *A. flavus* according to the standard methodology. The amplification was performed on a total volume of 25 µL containing: 10X PCR buffer 1.5 µL, 50 mM $MgCl_2$ 1.0µL; 200 mM dNTP 1.0µL; 20 pM daflR-R primer 1.0µL; 20 pM aflR-f primer 1.0 µL; 2.5 unit Taq DNA polymerase 0.25 µL; 20 mg DNA Template 5.0 µl; and Millipore H_2O. PCR reactions were carried out in a thermocycler following the cycling program, including the initial denaturation cycle at 94°C for 5 min., the 35 cycles, each cycle including the denaturation step at 94ºC for 30 sec., annealing step at 55 for 60 sec, extension step at 72°C for 90 sec and a final extension step at 72ºC for 7 min. The temperature and salt conditions were optimized for low inputs of template DNA. The amplification products were purified on contricon 100 columns (Amican) followed by ethanol precipitation.

Table 1. Primer sets sequences designed for *aflR* gene DNA condition's reaction.

Primer pain	Gene	Primer sequence (5 -3)	Optimal annealing temp	PCR size
Omtl-F	aflP	GCCTTGCAAACACACTTTCA	55⁰ C	1490 bp
Omtl-R		AGTTGTTGAACGCCCCAGT		

Agarose gel electrophoresis

The two *A. flavus* isolate PCR products (50 µl) were detected using 1.5% agarose gel electrophoresis in 1 X TAE buffer and stained with ethidium bromide. The amplified DNA bonds were visualized under a UV light-trans-eliminator, and the size of expected DNA fragments was estimated based on a DNA ladder of 100 to 2000 bp (manufactured by Bioran).

DNA sequencing

PCR products of *A. flavus* isolates were purified using the QIA prep. spin miniprep kit protocol (QIAGEN) and purified DNA was sequenced using an automated DNA sequencer. The results were aligned to the DNA sequences of *A. flavus* isolates using Bio Edit version 7 software (www.Mbio-NCUs.Edu/bio. Edit). The nucleotide sequence of *A. flavus* isolates was compared to accessions of *Aspergillus* sp. available in the NCBI database using BLAST- algorithm to identify closely related sequences (http/WWW.NCbI.Nih.Gov). Dendrograms were constructed using the unweighted pair Group method with Arithmetic (UPGMA).

RESULTS

Pictorial identification of isolated *A. flavus*

The *A. flavus* from the incubated corn grains was observed using a stereomicroscope and was photographed in Fig (1). The growth of fungi was covering the infected part of the grains. These characters will help the analyst to identify the isolated fungi. Photographs from different magnifications support the descriptions. The growth of *A. flavus* on disinfected corn grains was characterized by immature and white mature heads

with other shades ranging from yellowish cream to green. Conidiophores were bearded with clear heads when the growth was white. They were long and hyaline, terminating in the form of bulbous heads. Conidia were globose to subglobose, usually rough, yellowish green in colour.

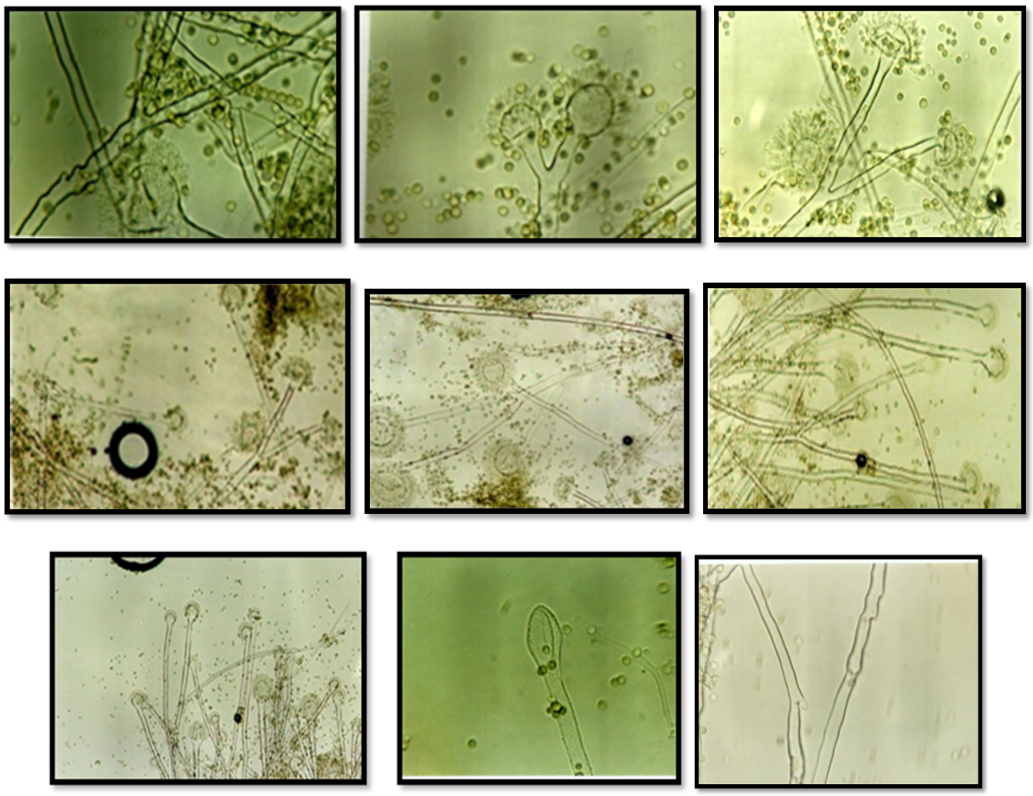

Figure 1. Photographs showing *A. flavus* treated (UV) and original (O) isolates infected corn seeds. Photographs illustrate conidiophores, conidial heads, and conidia detected at X400 magnification.

Mycotoxins production

The two *A. flavus* isolates (UV treated) and (O original) inoculated on corn grains were tested for aflatoxin production by high liquid chromatography technique (HPLC). Aflatoxin was produced by the toxigenic fungi, *A. flavus* (UV) and (O) isolates at 35.210 and 195.75 ug /kg of grains, respectively. The aflatoxin types AfB1, AfB2, AfG1, and AfG2 produced by toxigenic fungi differed in quality and quantity based on fungal isolates. It was found that *A. flavus* (UV) produced AfB1 at 35.250 ug/kg of seeds. In comparison, *A. flavus* (O) produced AFB1, AFB2, AFG1, and AFG2 at 45.74, 10.4, 15.24, and 135.20 ug/kg of grains (Table 2).

Molecular characters of aflR gene

The integrity and quantity of purified total DNA of the *A. flavus* isolates (UV) and (O) were confirmed by agarose electrophoreses and visualised using a UV spectrophotometer at an absorption ratio of A_{260}/A_{280} (1.8 and 1.5), respectively. The concentration of DNA was 80 and 95 ug/5 g mycelium, respectively. These results indicated the high yield and purity of the isolated DNA.

The aflatoxin DNA gene (aflR gene) from the two *A. flavus* isolates were amplified using PCR and specific primer sets. The amplified aflR gene size was analyzed using agarose gel electrophoresis and compared to the standard DNA leader. The amplified fragment of the aflatoxin gene PCR product had an expected size of ≈ 1350 bp for two *A. flavus* isolates (Fig. 2).

Table 2. The quantitative and qualitative analysis of the production of aflatoxins and fumonisins the toxigenic fungi on corn grains.

Aflatoxins		Standard	*A. flavus* (UV)	*A. flavus* (O)
	RT	9.633	9.617	9.678
	Area	66292517	214577	445546
AfB1	Height	2695050	9409	18570
	Amount		35.250	45.75
	Units			
	RT	19.009	-	19.000
	Area	31624309	-	
AfB 2	Height	1020489	-	
	Amount		-	10.40
	Units		-	
	RT	7.401	-	7.420
AfG1	Area	35157208	-	112227
	Height	1833710	-	6167
	Amount		-	15.35
	Units		-	
	RT	13.265	-	13.506
	Area	21254335	-	
AfG2	Height	925554	-	
	Amount		-	124.250
	Units		-	-

Af =Aflatoxin types B1, B2, G1, G2.

Nucleotide sequence analysis

The PCR products of aflRgene were eluted from agarose gel using the gel DNA extraction kit. Amplicons forming the PCR were allowed for sequencing reactions through the cycle sequencing method. The sequencing was done from the forward direction at Mocrogen 3730X l6-1518-009 Korea. Primers in either the forward or reverse direction were easily identified in each segment of the sequence and were easily used to assemble the individual sequence. The sequences obtained for each primer for each isolate had sufficient overlap between the bands used to form a single continuous sequence (contig). The nucleotide sequences similarity among two *A. flavus* isolates were detected, and a phylogenetic tree revealed an 85% (moderate) degree of similarity among them (Fig.3). The partial nucleotide sequences (1335 and 1345 bp) from the DNA of the two *A. flavus* (UV and O) isolates were determined to identify any relationship with other recommended aflR gene *Aspergillus* strains registered in GenBank (Fig.4).

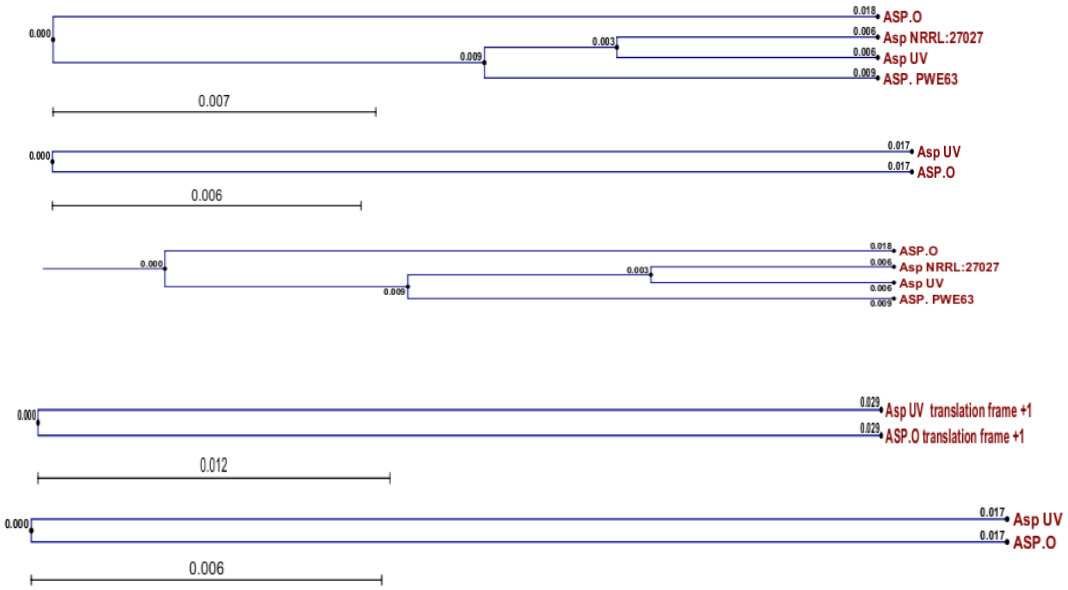

Figure 2 and 3 Phylogenic tree of aflatoxin gene for two *A. flavus* (Asp. UV and Asp. O) infected corn seeds based on the nucleotide sequences

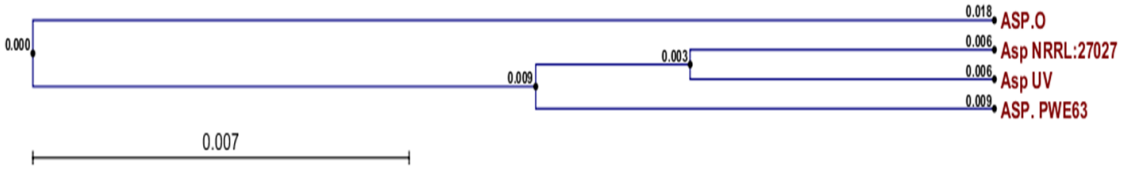

Figure 4. Phylogenetic tree of aflatoxin gene DNA for two *A. flavus* (Asp. UV and Asp. O) with corresponding two *Aspergillus* sp. isolates registered in GenBank.

Translation of aflatoxin gene DNA sequences

The aflatoxin gene of *A. flavus* isolates (UV and O) translation product was predicted from the universal code of aflatoxin gene nucleotide sequences: 445 (UV) and 448 (O) amino acids starting with methionine (M) for both *A. flavus* isolates (UV and O). The maximum open reading frame (ORF) starts at AA position 1 (maybe DNA position 1 of the aflatoxin gene for two *A. flavus* isolates). The molecular weight differed among the sequences at 47.502 and 47.625 K. Da for-aflatoxin gene sequences of UV and O isolates, respectively.

The partial aflatoxin gene amino acid sequences for two *A. flavus* isolates were aligned using the DNAMAN program (Wisconsin, Madison, USA). The alignment revealed the similarity among the two isolates. Seven sites differed between *A. flavus* isolates (Fig. 5).

Figure 5. Phylogenic tree of aflatoxin gene translated amino acids of two *A. flavus* (Asp. UV and Asp. O) infected corn seeds based on the nucleotide sequences.

The similarity index of the two *A. flavus* isolates (445and 448 amino acids of *aflR* gene DNA) were done to determine the relationship with other recommended *aflR* gene *Aspergillus* strains registered in GenBank (Fig.6). They were presented in a dendrogram and homology matrix. A phylogenetic tree of amino acid sequences for two isolates revealed 92% similarity (high degree).

Figure 6. Multiple sequence alignment of aflatoxin gene translated into amino acids for the two *A. flavus* (Asp. UV and Asp. O) and two *Aspergillus* sp isolates published in GenBank based on the nucleotide sequences.

Nucleotide and amino acids diversity

Nucleotide and amino acid sequence of aflatoxin gene revealed replacement bases among (UV) and (O) *A. flavus* isolates (fig.,7 and 8). The replacements were found at 39 sites, with 2.7% of nucleotide sequence dissimilarity among the two *A. flavus* isolates (UV and O). Amino acid site replacements were 7 with a percent dissimilarity of 1.50 % of amino acid sequences among two isolates (UV and O).

Figure 7. Alignment and replacement sites of nucleotide bases (39 bp) in nucleotide sequences of aflatoxin gene for *A. flavus* isolates.

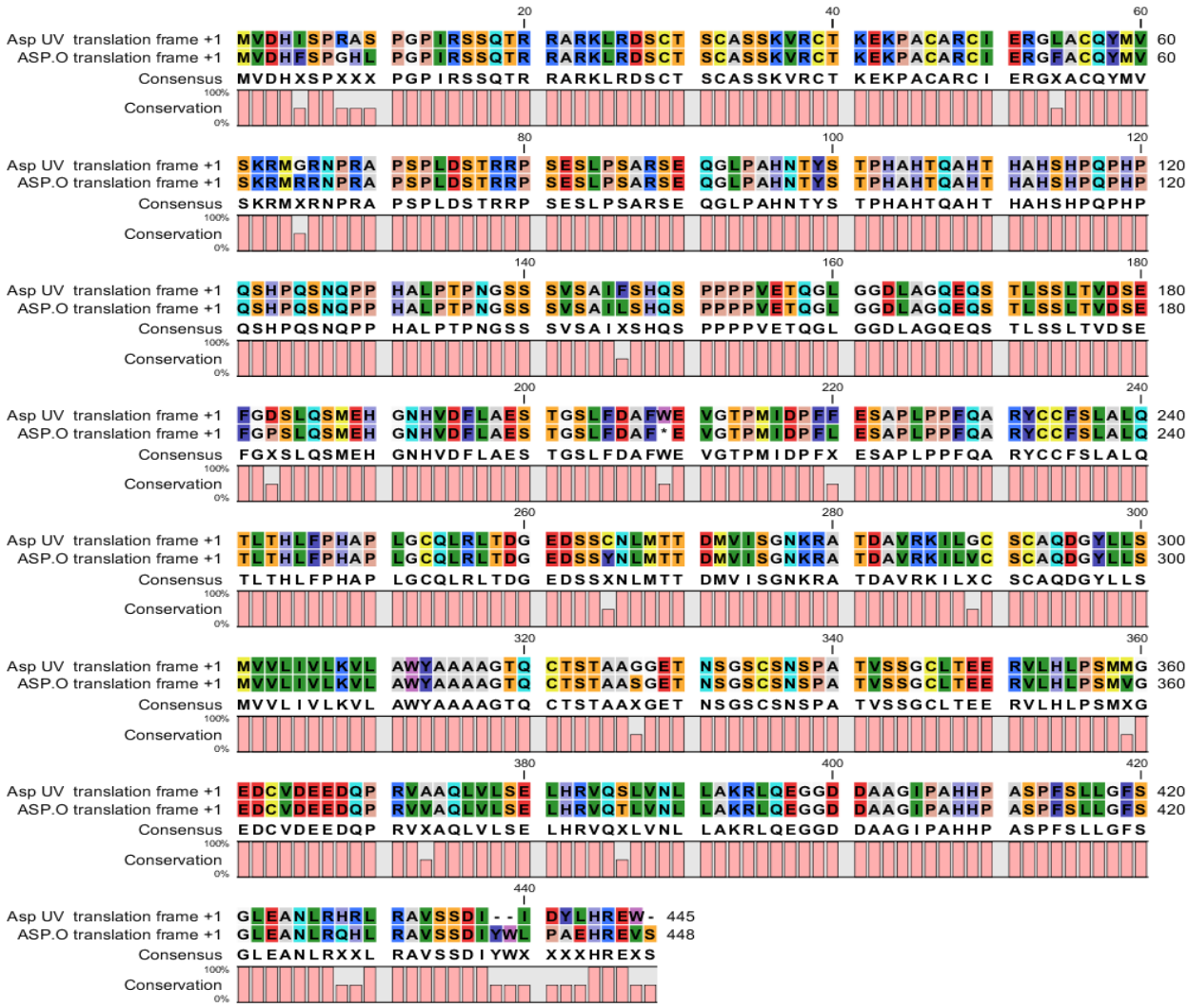

Figure 8. Alignment and replacement sites in protein sequences (7 amino acids) of aflatoxin gene for *A. flavus* isolates.

DISCUSSION

The total number of fungi isolated on PDA media was lower in sterilized maize grains than in non-sterilized maize grains. Similar results were reported by El-Nagerabi et al. (2000), who found that *Aspergillus* sp. was the most common isolated species, followed by *Rhizopus*, *Alternaria*, *Fusarium*, *Emericella*, *Drechslera*, *Cladosporium*, *Pencillium*, and *Pythium*. Many fungal species regularly associated with corn grains can infect developing seeds and still attach to the parent plant. This was demonstrated by isolating fungi from grains collected before maize grains were mature.

The identification process was carried out first by a manual method based on the naked eye at different magnifications using a stereomicroscope. To identify each species of *Aspergillus*, isolates were grown in a specific Czapek agar medium. The morphological structure of the genus *Aspergillus* depends on the head, stipe, vesicle, metulate, and conidia, according to Booth (1972) and Maren and John (1988), respectively. Colonies of *Aspergillus* consist of heads in different colors; in a few cases, some colonies showed the same color, but when viewed under the microscope, the distinction was seen in the type of head.

The two *A. flavus* isolates inoculated on corn grains were tested for their ability to produce aflatoxin by high liquid chromatography technique (HPLC). Aflatoxin concentrations were produced in UV (treated isolate) and O (original isolates) at 35.210 and 195.75 ug/kg of grains, respectively. The aflatoxin types AfB1, AfB2, AfG1, and AfG2 differed in quality and quantity based on the fungal isolate. It was found that, *A. flavus* (UV) produced AfB1 (35.250 ug/ kg seeds). In comparison, *A. flavus* (O) produced AFB1, AFB2, AFG1, and AFG2 with 45.74, 10.4, 15.24, and 135.20 ug/kg grains, respectively (Boubakar 2013). Not all strains of *A. flavus* and *A. parasiticus* produce aflatoxin; *A. parasitica* strains generally produced aflatoxin B1, B2, G1 and G2, while *A. flavus* produce B1 and B2 aflatoxin (Diener et al., 1987). In the current study, the total concentration of aflatoxin B1, B2, G1, and G2 was 205.75 ug /kg of seeds. It is well-documented that aflatoxin has a carcinogenic effect. The DNA genomes of A. *flavus* isolates (UV and O) was used as a PCR template to amplify the FIR gene. The IR gene, with an expected size of 1335 bp, could be synthesized using the internal primer sets (FG1 and rp2) to confirm its mutual specificity with *A. flavus*. Genetic variation among isolates was also detected. The substitution of nucleotide and amino acid sequences was 0.89% and 1.57% for UV and O types, respectively. Most of the 25 identified genes clustered within a specific 70-kb region of the fungal genome, they were shown to be involved in aflatoxin synthesis (Bhatnagar et al., 2003; Yabe and Nakajima, 2004). Among them, the aflR gene encodes a key transcriptional regulator of the aflatoxin synthesis gene (Curry et al., 2000). AflR is a regulatory gene for aflatoxin synthesis. It is called aflR-2 in A. flavus, which encodes a regulatory factor (AFLR protein).

In the current study, PCR was used to identify the molecular diversity among the *A. flavus* isolates by amplifying and sequencing the aflR gene, producing an aflR gene PCR product of 130 bp. The nucleotide sequence, the number of amino acids, and the amino acid sequence differed among the isolates. The DNAs of *A. flavus* isolates appeared to exhibit low levels of similarity to aflR gene since low amounts of PCR or no PCR products. Increased expression of aflatoxin biosynthetic genus in the transformant containing an additional copy of aflR might result from an elevated basal level of AFIR, allowing it to overcome nitrate inhibition of aflatoxin; in addition, aflR gene is involved in the regulation of multiple parts of the aflatoxin biosynthesis pathway (Chang et al., 1995). On aflR gene mutation in *A. flavus,* all the aflatoxin genes' expression was turned off or significantly reduced (Woloshuk et al., 1994). In addition, Flaherty and Payne (1997) demonstrated that altered and elevated transcription of aflR leads to clavated and altered gene expression pathways and higher levels of aflatoxin production.

Sequence analysis of the aflR gene from *A. flavus* revealed evolutionary sequence differences between isolates. These results indicated that some metabolites could be useful in distinguishing species efficiently and accurately. In addition, we measured the isolates sequence similarity by amplifying and sequencing the aflR gene. Lee et al. (2006) demonstrated that the aflR gene cannot be amplified from some strains of *A. oryzae* and *A. sojae*. In strains of *A. oryzae* from which the aflR gene can be amplified, the aflR gene showed differences from the aflR of toxigenic *A. flavus*. On the other hand, Hua et al. (2009) mentioned that the aflatoxin cluster genes aflR, aflJ, pks, and OmtB were expressed at higher levels in afla toxigenic isolates in comparison to strains that did not produce aflatoxins. In recent years, significant advances have been made in molecular diagnosis technology, especially in developing rapid and sensitive methods for detecting plant pathogenic fungi.

PCR, is widely used as a powerful molecular tool for identifying plant microorganisms. Due to its high sensitivity, this technique is used to identify the DNA of organisms, which are difficult to cultivate or identify

biologically. The ability to amplify DNA from crude mycelia preparations is essential in identifying fungi from the plant material. The primary advantage of using PCR is that it requires only small amounts of test material, and the technique is applicable even to partially degraded materials of poor quality Therefore, we need to develop detection procedures using PCR and detect sequences in the Target DNA region to design specific primers. By using PCR technique, strains, pathotypes, species or higher classes of microorganisms can be identified, provided that specific oligonucleotide primers are available for these strains. This method depends on the alignment of sequences from target and non-target organisms and identifying primers that do not match non-target organisms and have sufficient matching to prepare and amplify all target organisms effectively.

Detecting fungal pathogens from seeds involves culturing organisms from the infected seeds, extracting the DNA from the culture, and subjecting it to PCR. Despite the feasibility of the PCR method, it is yet to be commonly used in seed health testing. More research must be done before the technique can be adapted and routinely used in seed health testing. There are several variables within PCR itself, which must be optimized for each primer-species combination. From the quarantine point of view, it is also essential to know whether the pathogen detected in the seed by PCR method is viable since PCR is a highly sensitive technique; it detects even dead fungi and spores, which are of no pathological importance. Thus, PCR has many potential uses in analyzing seed-borne fungal pathogens. Identifying the type and species of pathogens and detecting fungal pathogens that are difficult to culture or identify is essential. The technique will significantly help monitor seed health in the international seed trade (quarantine), domestic seed certification programs (seed testing laboratories), and research laboratories. There are already many examples of PCR-based assays developed for detection of fungi in plant pathology, but the reports on their use in specific detection of seed borne fungi are limited (Mahthur and Olga, 2003, Caceres *et al*.,2020). Finally, it could be recommended that sterilization methods using ultraviolet rays be reviewed to detect the quality of stored seeds, and their impact on human and animal health must be further investigated.

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Conflict of interest statement

We declare that we have no conflict of interest.

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