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Isolation and identification of fungi associated with cotton seeds and investigation of aflatoxigenic *Aspergillus flavus*

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Abstract

Objective

This investigation targeted to isolate and identify fungi contaminating seeds of two long-term stored cotton cultivars and to identify which isolate generated the greatest level of aflatoxin, thereby evaluating potential risks to seed safety.

Materials and methods

Two cotton cultivars, *Ashura* and *Lashta*, stored for almost three years, were gathered in sterile bags, transported to the laboratory, and stored at 4 °C until analysis. Fungal isolation was carried out by surface plating on potato dextrose agar and malt extract agar with incubation at 25-28 °C. Colonies were described by macroscopic morphology and microscopic examination applying lactophenol cotton blue staining. Ammonia vapor exposure acted as a fast qualitative experiment for aflatoxigenic *Aspergillus* strains. Aflatoxin making by isolates was quantified applying an enzyme-linked immunosorbent assay (ELISA). Molecular identifying of the greatest aflatoxin-generating isolate was carried out by PCR amplification of the ITS region applying standard primers and genomic DNA extracted based on producer protocols.

Results

A total of 285 fungal isolates demonstrating multiple genera were regained from the two cottonseed samples. The *Ashura* sample produced 150 isolates, while *Lashta* produced 135. The dispensation of recognized taxa was *Aspergillus niger* (48.00%), *Rhizopus* spp. (44.56%), *Aspergillus flavus* (7.01%), and *Penicillium* spp. (0.35%). Three *A. flavus* isolates generated measurable aflatoxin under culture situations, with concentrations ranging from 27.0 ng·mL^{-1} to 42.0 ng·mL^{-1}. The isolate with the greatest aflatoxin product (42.0 ng·mL^{-1}) was affirmed as *Aspergillus flavus* by PCR and was in line with morphological identifying; this isolate has been deposited with a unique accession in GenBank.

Conclusions

Extended storage of cotton seeds can favor colonization by diverse fungal communities, containing aflatoxigenic *A. flavus* strains. Routine surveillance of stored seed lots, progressed storage hygiene, and aimed mitigation strategies are recommended to ensure seed safety and to reduce related health and economic risks.

Keywords: aflatoxins, *aspergillus flavus*, cotton seeds, fungal pollution

Paper Type: Research Paper.

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Introduction

Cotton (*Gossypium hirsutum* L.) is one of the most momentous agricultural and fiber crops worldwide, belonging to the family *Malvaceae* (Al-Zubaidi, 2021; Salih et al., 2019). Fungal pollution is a great reason of seed and grain deterioration through storage, consequencing in meaningful losses in product and quality (Abdullah et al., 2025). Between these pollutants, storage fungi, exclusively *Aspergillus flavus* and *A. parasiticus*, are of great worry because of their

capability to generate aflatoxins, highly toxic and carcinogenic secondary metabolites liable for the damaging reactions of moldy feed and food (El-Naghy et al., 1991). Different genera of fungi, containing *Aspergillus*, *Alternaria*, *Fusarium*, and *Penicillium*, are well-known mycotoxin generaters. Mycotoxins are secondary metabolites that can adversely influence on animal and human health even at low concentrations. Based on the Food and Agriculture Organization (FAO), mycotoxin pollution influences on almost 25% of the world's food supply (Al- Nuaimy, 2024; Nazhand et al., 2020; Gbashi et al., 2020; Areo et al., 2023). Other reports estimate that between 60% and 80% of crops are damaged globally because of mycotoxin pollution. Environmental factors like temperature, humidity, and substrate conformation perform a key role in fungal growth and toxin biosynthesis (Eskola et al., 2020). Climate change has further expanded the geographic range of *Aspergillus* species, by growing the risk of pollution in regions previously attended safe (IARC, 2015). While numerous investigations have studied aflatoxin pollution in laboratory patterns, humans, and livestock (Magnussen et al., 2013; Zhang et al., 2017), study on fungal pollution of Iraqi cotton varieties stays restricted. Previous investigations in Iraq have firstly centralizeed on wheat and other cereals. Traditional biochemical procedures cannot reliably distinguish between closely related microorganisms like bacteria, fungi, and viruses (Ahsani et al., 2010; Mohammadabadi et al., 2004; Khabiri et al., 2025). In contrast, polymerase chain reaction (PCR) proposes a fast, sensitive, and particular molecular approach (Shahdadnejad et al. 2016; Mohammadabadi et al. 2024) for microbial identifying, supplying outcomes within hours (Mohammadabadi et al., 2011; Khabiri et al., 2023; Mohammadabadi et al., 2025). Therefore, the present investigation targeted to isolate and identify seedborne fungi from two long-term stored cotton cultivars, quantify aflatoxin levels generated by toxigenic isolates, identify the most potent aflatoxigenic strain, and evaluate potential risks to the safety of stored cotton seeds.

Materials and methods

Sample collection: Cotton seed samples were derived from the Cotton Research Unit of the Northern Technical University (Mosul, Iraq), which supplied seeds from two broadly cultivated varieties, *Ashur* and *Lashta*. The seeds had been stored for almost three years under standard storage situations. Samples were without delay placed in sterile plastic bags, labeled, and transported to the laboratory for mycological analysis. Upon arrival, the samples were kept at 4 °C until further examining to prevent additional microbial growth or deterioration.

Seed safety testing: Two representative seed samples of the *Ashur* variety were applied to evaluate seed viability and fungal pollution. Amoxicillin was applied as an antibacterial agent to suppress bacterial growth. Ten seeds were put on each plate including Potato Dextrose Agar

(PDA) medium, with a total of 100 seeds examined. The plates were incubated at 25 ± 2 °C for seven days under alternating 12-hour light and dark cycles. After incubation, fungal colonies emerging from the seeds were tested and documented based on their growth traits and phenotype.

Determination of fungal occurrence: The percentage occurrence of each fungal species was determined to identify the prevalence of infection applying the subsequent formula:

Frequency (%) = (Total number of isolates of each species ÷ Total number of fungal isolates) × 100

This analysis aimed clarify which fungi were most often related to the cotton seed varieties and evaluate their relative levels of pollution.

Diagnosis of fungal isolates: Fungal isolates were subcultured on weak aqueous agar medium to obtain clear microscopic images of sporophores and spores. A 7 mm diameter disc of each pure colony was aseptically transferred applying a cork borer sterilized by immersion in 70% ethanol, flaming, and cooling. The discs were placed on the surface of glass plates containing the same weak agar medium. After suitable growth, a small square section of agar containing a expanding colony was removed and mounted on a glass slide with a drop of lactophenol cotton blue stain (HiMedia, India). The slide was gently heated to partially liquefy the agar, permiting the hyphae and spores to spread evenly. The cover slip was carefully pressed to remove air bubbles and raise microscopic visibility. Slides were examined under a combination microscope to observe conidial structures and spore morphology for species-level identifying.

Identification of toxigenic *Aspergillus flavus* applying ammonia vapor: Twenty isolates of *A. flavus* derived from the cotton seeds were screened for aflatoxin making applying the ammonia vapor procedure as characterized by Abd-Elsalam et al. (2023). Mycelial plugs (5 mm diameter) from actively growing colonies were inoculated onto Coconut Agar Medium (CAM) and incubated at 28 °C in darkness for seven days. After incubation, the plates were inverted, and one to two drops of concentrated sodium hydroxide (NaOH) were applied to the inner surface of the Petri dish lid. Within five minutes, aflatoxigenic colonies expanded a pink to red coloration, whereas atoxigenic isolates stayed unchanged.

Purification of fungal isolates: Fungal isolates were purified applying the single-spore isolation technique. A loopful of each fungal colony was suspended in 10 mL of sterile distilled water containing one drop of Tween 80 to disperse the spores evenly. The suspension was vortexed thoroughly, and tenfold serial dilutions (10^{-1} and 10^{-2}) were prepared. One milliliter of the diluted suspension was spread dropwise onto the surface of water agar plates, with each drop positioned within a 1 cm² marked grid. Plates were incubated for 2-3 hours to permit individual spores to germinate. Germinating single spores were seen under a stereomicroscope and aseptically transferred to PDA plates to establish pure colonies for further analysis.

Screening of isolates for toxigenicity applying ELISA: Dried mycelial mats (almost 250 mg) from purified fungal cultures were gathered for toxin quantification. Samples were stored under dry, dark, and low-temperature situations to prevent degradation. The samples were finely ground to obtain a homogeneous powder. Almost 200 mg of each sample was isolated with 20 mL of 70% methanol subsequent the protocol supplied with the ELISA kit (Sunlong Biotech Co., China). The mixture was shaken vigorously, and the extractive was filtered to remove particulate matter. Aflatoxin concentrations were quantified applying the ELISA procedure (Rayto Instruments, Germany) based on the producer's instructions (Li et al., 2016). Optical density was calculated at 450 nm applying a microplate reader. Standard curves were generated from known aflatoxin concentrations to identify toxin levels in the examined samples.

Molecular identification of the most toxigenic *Aspergillus flavus:* Molecular confirmation of the most toxigenic *A. flavus* isolate was managed at the Technical Research Center (TRC), Northern Technical University. Genomic DNA was isolated applying the Wizard Genomic DNA Purification Kit (Genesis, Taiwan) subsequent the producer's protocol, with minor optimization for fungal samples. The Internal Transcribed Spacer (ITS) region of the ribosomal DNA was amplified applying universal fungal primers ITS-1 (5′-TCC GTA GAA CCT GCG G-3′) and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′).

Polymerase Chain Reaction (PCR) was carried out in a 25 µL reaction mixture containing template DNA, primers, deoxynucleotide triphosphates, buffer, $MgCl_2$, and Taq DNA polymerase. Amplification was carried out in a thermocycler under the subsequent situations: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min; followed by a terminal extension at 72 °C for 7 min. Amplified DNA fragments were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized under ultraviolet (UV) illumination applying a transilluminator. Gel images were captured with a Canon digital imaging system (USA). The derived ITS sequences were in comparison to reference sequences in the NCBI GenBank database to affirm species identity.

Results

Isolation and identification of fungi: The safety of cotton seed samples from two cultivars, *Ashur* and *Lashta*, was evaluated applying the agar plate procedure on Potato Dextrose Agar (PDA) medium. A total of 285 fungal isolates belonging to different genera and species were regained (Table 1; Figures 1 and 2).

Table 1. Fungi extracted from the two cotton seed varieties on PDA medium

Fungal frequency	Isolation %	Isolated fungal species	Seed samples/cultivar
66.66	100	*Rhizopus* spp.	Ashur
33.33	50	*Aspergillus niger*	
-	-	Total = 150	
6.44	87	*Aspergillus niger*	Lashta
20	27	*Rhizopus* spp.	
14.81	20	*Aspergillus flavus*	
0.74	1	*Penicillium* spp.	
-	-	Total = 135	

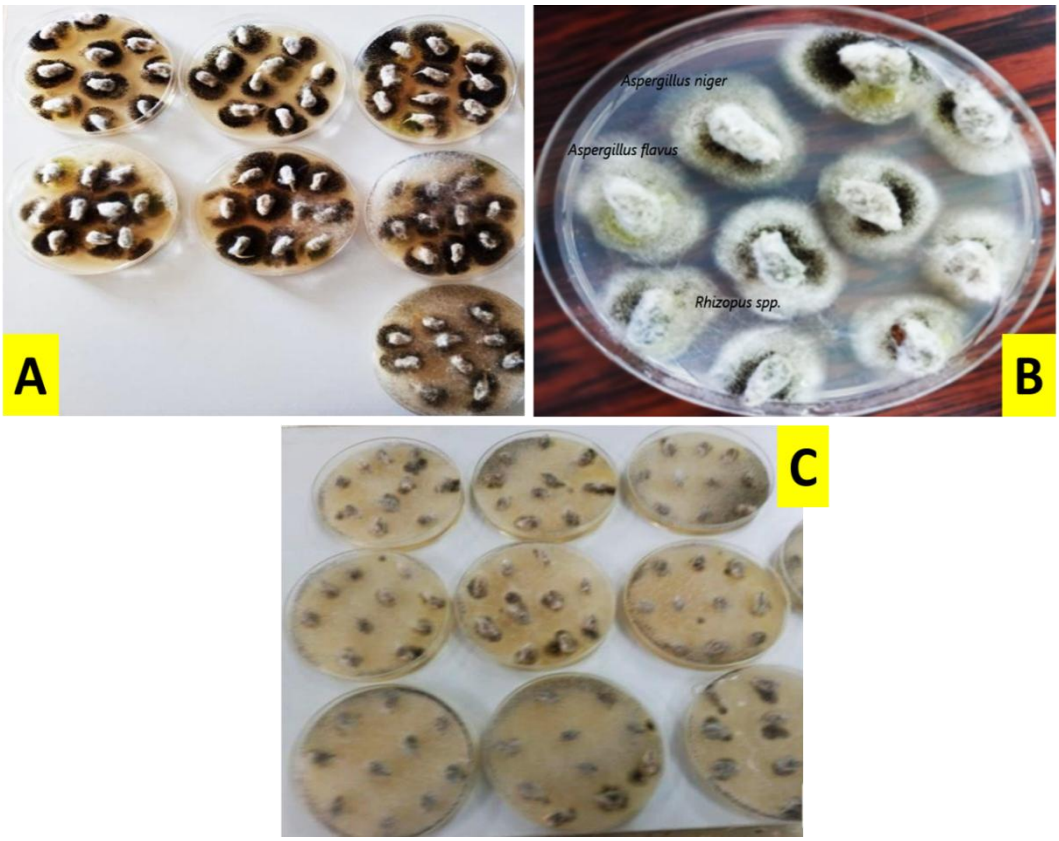

Figure 1. Macroscopic appearance of fungi related to cotton seeds on PDA medium: (A) *Lashta* seeds showing mixed fungal growth; (B) colonies of *A. flavus*, *A. niger*, and *Rhizopus* spp.; (C) fungal growth from *Ashur* seeds

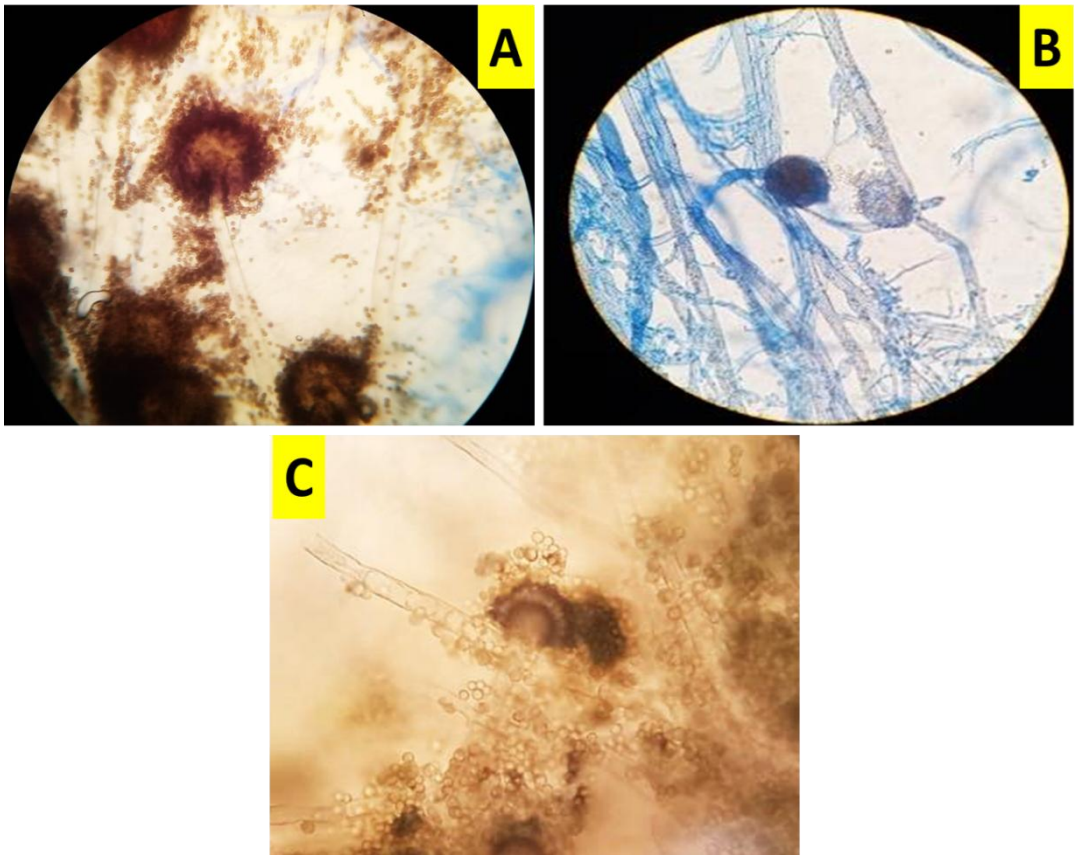

Figure 2. Microscopic examination (40× magnification): (A) *A. niger*; (B) *Rhizopus* spp.; (C) *A. flavus* extracted from cotton seeds

Frequency of fungal infection: The frequency of fungal occurrence was calculated to evaluate the susceptibility of the two cotton cultivars to seed-borne fungi. The *Ashur* variety exhibited the greatest level of pollution, yielding 150 fungal isolates (52.63%), while *Lashta* generated 135 isolates (47.36%) (Table 2).

Table 2. Total number and frequency of fungi isolated from the two cotton seed varieties

Cotton variety	Total number of isolates	Frequency %
Ashur	150	52.63
Lashta	135	47.36

Between the extracted species, *Aspergillus niger* was the most frequent fungus, accounting for 48.07% of the total isolates. *Rhizopus* spp. ranked second (44.56%), followed by *A. flavus* (7.01%) and *Penicillium* spp. (0.35%) (Table 3).

Table 3. Frequency of fungi extracted from cotton seeds on PDA medium

Isolated fungi	Total number of isolates	Frequency %
Aspergillus niger	137	48.07
Rhizopus spp.	127	44.56
A. flavus	20	7.01
Penicillium spp.	1	0.35

Incidence of toxigenic isolates (Ammonia vapor test): The ammonia vapor assay disclosed that out of twenty *A. flavus* isolates derived from the *Lashta* variety, only three strains exhibited a positive color change, denoting aflatoxin making potential. The staying seventeen isolates were classified as atoxigenic, as no color reaction occurred upon exposure to ammonia vapor.

Aflatoxin concentration identified by ELISA: Quantitative estimation of aflatoxin making was carried out applying enzyme-linked immunosorbent assay (ELISA). The three *A. flavus* isolates that examined positive in the ammonia vapor assay showed aflatoxin concentrations of 42.0, 34.0, and 27.0 ng mL^{-1}, respectively (Figure 3). These results affirm variable toxigenic potential between the isolates, with one strain generating markedly higher levels of aflatoxin.

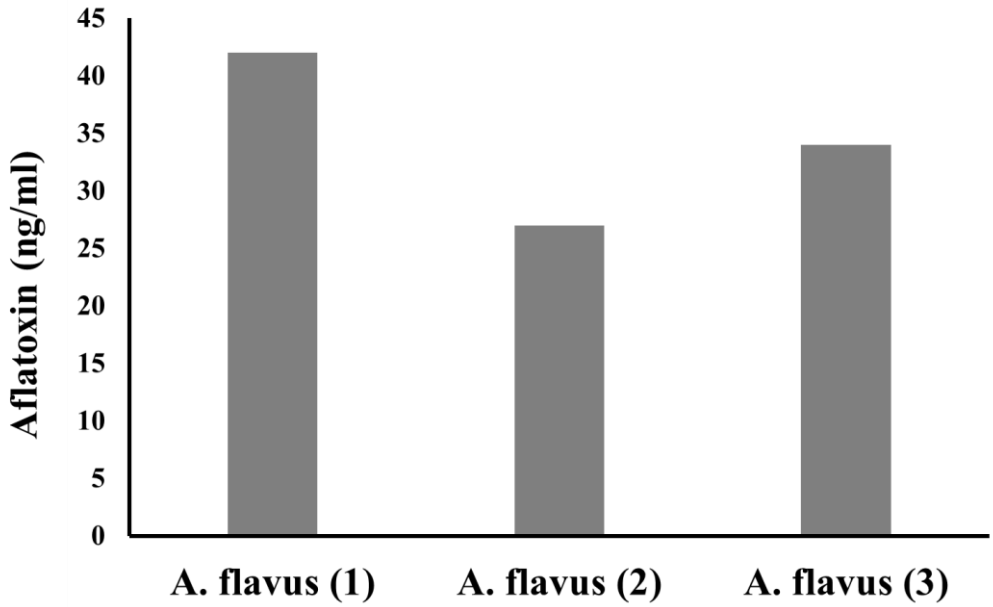

Figure 3. Concentrations of aflatoxin (ng mL^{-1}) found by ELISA in the three *A. flavus* isolates derived from cotton seeds

Molecular diagnosis of *Aspergillus flavus* isolate 3 applying PCR: Polymerase chain reaction (PCR) analysis was employed to affirm the molecular identity of the most toxigenic *A.*

flavus isolate. The internal transcribed spacer (ITS) region was amplified applying universal fungal primers ITS1 and ITS4, which target the ITS1-5.8S-ITS2 region of ribosomal DNA. The amplification generated a separate band in line with *A. flavus* when visualized on 1.5% agarose gel under ultraviolet illumination. Sequence analysis of the amplified fragment affirmed that the isolate was *Aspergillus flavus* and showed high similarity to known reference sequences in the NCBI GenBank database. The strain was designated Aspergillus flavus J-K-AL-Turjuman and assigned the unique accession number PV114988.1. This identifying validates the morphological and microscopic results and affirms the purity and genetic separateiveness of the isolate.

Discussion

The variation in fungal frequency seen between the examined cotton seed varieties may be attributed to differences in seed moisture content, seed coat integrity, and varietal susceptibility to fungal infection, as well as to variations in environmental situations through storage (Al-Abdalall, 2008). The activity and proliferation of storage fungi generally improve with higher grain temperatures and longer storage phases. Abdel Salam et al. (2023) announced that *Penicillium* sp. (16.74%), *A. niger* (36.02%), and *A. flavus* (19.29%) were the predominant fungi contaminating cotton seeds, results that are equivalent to the outcomes of the present investigation. Similarly, Aly et al. (2008) documented that non-surface-sterilized cotton seeds were often colonized by *A. flavus*, *A. niger*, *Aspergillus* sp., *Chaetomium* sp., *Cladosporium* sp., *Fusarium moniliforme*, *F. oxysporum*, *Nigrospora* sp., *Penicillium* sp., *Rhizopus stolonifer*, *Stemphylium botryosum*, *Trichoderma* sp., and *Alternaria alternata*. These outcomes collectively demonstrate the diverse fungal flora related to stored cotton seeds and emboss the dominant role of *Aspergillus* and *Rhizopus* species. Mohana et al. (2011) analyzed 30 seed samples and recognized 12 fungal species from 8 genera, containing *A. flavus*, *A. niger*, *Rhizopus* spp., *Penicillium* spp., *Fusarium moniliforme*, *F. oxysporum*, *F. pallidoroseum*, *Phoma exigua*, *Stagonospora nodorum*, *Bipolaris spicifera*, and *Cladosporium herbarum*. The most prevalent species were *A. flavus* and *F. moniliforme*. These outcomes are in line with the present results, affirming *A. flavus* and *A. niger* as great pollutants of stored cotton seeds. Variability in aflatoxin making between *A. flavus* isolates has been broadly documented and is firstly attributed to genetic diversity within the species (Al-Nuaimy and Abed, 2021 and 2024; Saadallah *et al.*, 2023; Abdullah et al., 2024; Al-Rejaboo, 2025). Not all isolates contain a complete aflatoxin biosynthetic gene cluster, and some harbor mutations that render the pathway nonfunctional (Adhikari et al., 2016). Consequently, the presence of *A. flavus* does not necessarily illustrate aflatoxin pollution. Environmental situations further impression toxin making, high temperatures and moderately dry air promote aflatoxin biosynthesis, while high humidity favors fungal growth

but often suppresses toxin agglomeration (Kumar et al., 2021). The reactions of climate change, containing rising global temperatures and extended droughts, have participated to the northward expansion of *A. flavus* and improved aflatoxin pollution in previously unaffected regions (Al-Tamimi et al., 2022). Therefore, perception environmental and genetic factors affecting aflatoxin making is crucial for expanding effective control strategies. Aflatoxin biosynthesis involves a compound cluster of over 25 genes, containing key regulatory genes (*aflR* and *aflS*) and different constructional genes like *aflD*, *aflM*, and *aflP*. Full activation and functionality of these genes are necessary for toxin making. Mutations or deletions, exclusively in the *aflR* regulatory gene—can result in atoxigenic phenotypes even when fungal growth stays robust (Demirev et al., 2006). Some isolates may carry quiescent or transcriptionally inactive biosynthetic genes, preventing toxin expression in spite of the presence of the genetic framework (Matsushima et al., 2001). Environmental parameters like temperature, substrate conformation, and oxygen availability further modulate the regulation of aflatoxin biosynthetic genes. Even under ideal laboratory situations, only isolates with complete and functional gene clusters can synthesize aflatoxins (Amaike and Keller, 2011). For instance, Mohankumar et al. (2010) announced that seventeen *A. flavus* isolates from maize grown across numerous agroecological zones in Tamil Nadu, India, exhibited a broad range of aflatoxin B_1 making (1.9-206.6 ng/mL) when analyzed applying indirect competitive ELISA. This variability underscores the combined impression of genetic potential and environmental situations on toxin biosynthesis. Overall, the current results align with previous reports, denoting that *A. niger* and *Rhizopus* spp. are the most common seed-borne fungi in cotton, while *A. flavus* represents a smaller yet critical portion because of its toxigenic potential. The identifying of only a few aflatoxigenic *A. flavus* isolates proposes that toxin making is not ubiquitous within the species but rather strain-dependent, necessitating molecular and biochemical verification in risk evaluation investigations.

Conclusion: The isolation of multiple fungal species from both *Ashur* and *Lashta* cotton seed varieties embosses the susceptibility of stored seeds to fungal colonization. The low frequency of aflatoxigenic *A. flavus* isolates, together with their genetic diversity, emphasizes the requirement for direct aflatoxin screening rather than assuming toxicity based on fungal presence alone. These results stress the importance of routine monitoring of stored cotton seeds to detect potential mycotoxin pollution, thereby ensuring seed safety and minimizing health and economic risks. Furthermore, implementing best practices in seed drying, storage, and handling—along with expanding resistant cultivars via breeding or genetic engineering—could meaningfully reduce fungal infection and aflatoxin pollution in cotton seed storage systems.

Author contributions

Conceptualization: Y.N.M.A.; Methodology: M.M.T.A. and J.K.A.-T.; Investigation: M.M.T.A.; Formal Analysis: J.K.A.-T.; Resources: M.M.T.A.; Writing—Original Draft Preparation: Y.N.M.A.; Writing—Review and Editing: Y.N.M.A.; Supervision and Project Administration: Y.N.M.A. All authors have read and approved the terminal version of the manuscript.

Data availability statement

The data supporting the results of this investigation are available from the corresponding author upon reasonable request.

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Ethical considerations

This investigation did not involve humans or animals. All experimental procedures were managed in accordance with institutional biosafety regulations and good laboratory practice (GLP) guidelines.

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Conflict of interest

The authors declare that they have no conflicts of interest relevant to this work.

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جداسازی و شناسایی قارچ‌های مرتبط با بذر پنبه و بررسی قارچ آفلاتوکسین‌زای *Aspergillus flavus*

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چکیده

هدف: این پژوهش با هدف جداسازی و شناسایی قارچ‌های آلوده‌کننده‌ی بذرهای دو رقم پنبه که به مدت طولانی در انبار نگهداری شده بودند، و همچنین تعیین جدایه‌ای که بیشترین میزان آفلاتوکسین را تولید می‌کند، انجام شد تا خطرات بالقوه برای ایمنی بذر ارزیابی شود.

مواد و روش‌ها: دو رقم پنبه، عاشورا و لاشتا، که حدود سه سال نگهداری شده بودند، در کیسه‌های استریل جمع‌آوری و به آزمایشگاه منتقل شدند و تا زمان آزمایش در دمای ۴ درجه سانتی‌گراد ذخیره گردیدند. جداسازی قارچ‌ها از طریق کشت سطحی بر روی محیط‌های *Potato Dextrose Agar* و *Malt Extract Agar* با انکوباسیون در دمای ۲۵ تا ۲۸ درجه سانتی‌گراد انجام شد. کلنی‌ها بر اساس ویژگی‌های ماکروسکوپی توصیف و سپس با استفاده از رنگ‌آمیزی *Lactophenol Cotton Blue* از نظر میکروسکوپی بررسی شدند. آزمون قرارگیری در معرض بخار آمونیاک به‌عنوان آزمایشی سریع برای شناسایی کیفی سویه‌های آفلاتوکسین‌زای *Aspergillus* به کار رفت. میزان تولید آفلاتوکسین توسط جدایه‌ها با استفاده از روش *ELISA* اندازه‌گیری شد. شناسایی مولکولی جدایه‌ی دارای بیشترین تولید آفلاتوکسین از طریق تکثیر ناحیه‌ی *ITS* با روش PCR و به‌کارگیری آغازگرهای استاندارد و DNA ژنومی استخراج‌شده بر اساس دستورالعمل تولیدکننده انجام گرفت.

نتایج: در مجموع ۲۸۵ جدایه‌ی قارچی از دو نمونه‌ی بذر پنبه به‌دست آمد که متعلق به چندین جنس مختلف بودند. نمونه‌ی رقم عاشورا ۱۵۰ جدایه و رقم لاشتا ۱۳۵ جدایه تولید کرد. توزیع گونه‌های شناسایی‌شده شامل *Aspergillus niger* (۴۸ درصد)، *Rhizopus* spp. (۴۴/۵۶ درصد)، *Aspergillus flavus* (۷/۰۱ درصد)، و *Penicillium* spp. (۰/۳۵ درصد) بود. سه جدایه‌ی *A. flavus* در شرایط کشت آزمایشگاهی مقادیر قابل اندازه‌گیری آفلاتوکسین تولید کردند که غلظت آن‌ها بین ۲۷ تا ۴۲ نانوگرم بر میلی‌لیتر متغیر بود. جدایه‌ای که بیشترین میزان آفلاتوکسین (۴۲ نانوگرم بر میلی‌لیتر) را تولید کرد، از طریق PCR به عنوان *Aspergillus flavus* تأیید شد و با شناسایی مورفولوژیک نیز مطابقت داشت؛ این جدایه با شماره‌ی دسترسی منحصربه‌فرد در پایگاه GenBank ثبت گردید.

نتیجه‌گیری: نگهداری طولانی‌مدت بذر پنبه می‌تواند موجب کلونیزه شدن آن توسط اجتماعات قارچی متنوع، شامل سویه‌های آفلاتوکسین‌زای *A. flavus* شود. پایش مستمر دسته‌های بذر ذخیره‌شده، بهبود بهداشت شرایط انبار و اجرای راهبردهای کاهنده‌ی هدفمند برای تضمین ایمنی بذر و کاهش خطرات بهداشتی و اقتصادی مرتبط توصیه می‌شود.

کلمات کلیدی: آفلاتوکسین‌ها، آلودگی قارچی، بذر پنبه، *Aspergillus flavus*

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