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Research Article

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Identification and Characterization of Non-aflatoxin Producing *Aspergillus* Species

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ABSTRACT
Aflatoxins are secondary metabolites formed by *Aspergillus* species, mostly *Aspergillus flavus* and *Aspergillus parasiticus*. Among the mycotoxins that contaminate agricultural products, AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFM_2 are the most toxic and carcinogenic. Not all species of *Aspergillus* produce aflatoxins; some strains are non-toxic. It is estimated that about 1.6 billion dollars of the global food crops are thought to be mycotoxin contaminated. AFs contamination in maize grains is 10.0 µg/kg in central Punjab, Pakistan. It is a major global challenge to manage *Aspergillus* infection, biocontrol methods are most effective and innovative by applying non-aflatoxigenic spores onto the field prior to harvest. The study aimed to estimate the incidence of aflatoxin and to identify non-aflatoxin *Aspergillus* species in four Tehsils of Multan district. The survey in Multan district revealed varying incidence percentage across tehsils, the highest occurrence of *A. flavus* (26.21%) was recorded in Tehsil Multan while lowest (13.45%) incidence was found in Tehsil Multan Saddar. The use of Coconut milk agar (CMA), *Aspergillus flavus* and *Aspergillus prasiticus* Agar (AFPA) and Potato dextrose agar (PDA) media in this study enabled sufficient growth, sporulation and pigmentation, allowing for a thorough identification of aflatoxin and non-aflatoxin producing *Aspergillus* isolates. *A. flavus* and *A. niger* isolates on CMA media under UV light at 365 nm were screened, 19 out of 20 isolates showed blue fluorescence while non-aflatoxigenic *A. niger* showed none. On AFPA media, *A. flavus* isolates indicated orange coloration underside their colonies which were aflatoxin producing and *A. niger* isolate did not show any coloration and considered as non-aflatoxin *Aspergillus* isolate. While on PDA media, the 7 days old isolates of *A. flavus* were detected through Ammonia Vapor Test (AVT) with a drop of concentrated ammonia hydroxide turned plum red indicating aflatoxin and *A. niger* showed no color change and confirmed as non-aflatoxin producing *Aspergillus* isolate. The use of selective media like CMA and AFPA were effective for morphological identification by distinguishing between aflatoxigenic and non-aflatoxigenic isolates when advance tools were accessible. Incorporating non-aflatoxin producing *A. niger* strain into crop management can reduce the risk of aflatoxin contamination, improving food safety and economic stability.
Keywords: *Aspergillus flavus, Aspergillus niger,* Disease incidence, UV light screening, AFPA media, Ammonia vapor test.

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INTRODUCTION
Aspergillus is one of the oldest and most prevalent genus of fungi, initially described by Pier Antonio in 1960 which belongs to the members of genus *Aspergillus*, a group of filamentous Deutromycetes (Samson *et al.*, 2014). They are named because of its spore bearing structure which resembles the Aspergillum, a sprinkler used in Roman Catholic churches to scatter holy water. *Aspergillus* was one of the most well-known and extensively researched mold families during Tom and Church released the first significant book on the genus in 1926. *Aspergillus* comprises approximately 446 species which are known and distinguished by their distinct spore bearing structure called the aspergillum (Houbraken *et al.*, 2020). Some species have teleomorphs (sexual states) that are usually seen

as cleistothecial with ascospores organized unusually within dehiscent asci. The genus is classified into different divisions. For example, *Aspergillus flavus* is classified under section *Flavi*. Notable mycotoxins producing fungi like *A. flavus* and *A. parasiticus* are found in this section and are economically very important (Frisvad *et al.*, 2005). These species are found throughout the environment and grow on plants, decomposed organic materials and inhabit a variety of environments, *A. flavus* germination and aflatoxin synthesis require relative humidity levels between 78 to 88% (Ribeiro *et al.*, 2006). The spores are found in soil, air that has high pathological, agricultural, industrial, pharmacological, scientific, and cultural significance (Paulussen *et al.*, 2017). They are distinguished by the presence of a spore-bearing component known as the conidia head and an unseptated basal foot called a "foot cell." Most of the species are differentiated according to their conidial shape. The phenotypic principles are still considered necessary in the taxonomy (Chi and Craven, 2016). Many of the *Aspergillus* genus are well-adopted to different environmental situation. The species can produce sexual ascospores that are persistent in environmental condition, also asexual conidia which are considered airborne and are capable of bearing extreme stress temperature (Wyatt *et al.*, 2015) *Aspergillus* species secrete many secondary metabolites like mycotoxins which are also produced by *Penicillum* and *Fusarium* and many more species. The agricultural goods are mostly associated with *A. niger*, *A. carbonarius* and *A. ochraceus* (Perrone *et al.*, 2007). One of the threatening specie such as *A. flavus* live on various waste products and agricultural commodities as saprobe (Yu, 2012). These fungi are frequently detected in many agricultural crops before and after harvesting, in storehouses including rice, wheat, corn, peanuts, and oilseed crops. *Aspergillus* species are found in different climate zones, however they are mainly found in mild regions between 16° and 35° latitude. (Klich, 2007). Most of the species are found in environment like terrestrial which can grow as saprophytes on the decomposed plants as mycelia on plant tissues, as conidia and sclerotia (wintering structures) in soil. The substances are known to be extremely hazardous, mutagenic, teratogenic, and carcinogenic. They have also been linked to the development of extrahepatic and hepatic cancer in humans (Santos *et al.*, 2010). Aflatoxin contamination can occur anywhere in the food chain, including in the field, during harvest, during handling, during shipping, and during storage (Giray *et al.*, 2007). Aflatoxigenic fungi have the ability to infect several agricultural goods, which can lead to aflatoxins contamination. Aflatoxigenic molds have also been discovered to be infesting major crops, such as maize, rice, and cottonseed, in several nations. Aflatoxin occurrence in foods and feeds is comparatively higher in tropical and subtropical areas because of the warm, humid weather that encourages the growth of fungi (Luttfullah and Hussain, 2011). Aflatoxin synthesis and *A. flavus* growth are dependent on various factors such as the kind of substrate, fungus species, moisture content, minerals, temperature, humidity, and physical damage to the kernels in natural substrates (Ribeiro *et al.*, 2006). The climate of Pakistan, which is warm and humid, is conducive to the growth of mycoflora, including *Aspergillus*, which can produce AFs as secondary metabolites (Ashraf *et al.*, 2023). There is a keen interaction of mycotoxins and climate which is often observed in Pakistan in different areas (Khan *et al.*, 2010). Toxic metabolites from a number of *Aspergillus* species contaminate grains agricultural commodities endangering the health of people and other animals with weakened immune systems (Kowalska *et al.*, 2017). A more recent study carried out in 2021 found biomarkers showing exposure to aflatoxins in 11% of children living in Pakistan Multan area (Nasir *et al.*, 2021). It has been proposed that non-toxic strains of *A. flavus* could act as biological control agents by competing with naturally occurring toxic strains of the *A. flavus* (Dorner, 2009). There are several strains of *A. flavus* and many more species, not all of them can produce aflatoxins. This is because some fungal strains may lose their ability to produce aflatoxin and become unpredictable in their production (Criseo *et al.*, 2001). *A. flavus* comprises of a vast variety of strains that can be found all over the world, including both aflatoxin and non-aflatoxin. The introduction of non-aflatoxin strains into the field is necessary to reduce the toxicity by making food fit for consumption (Bhatnagar-Mathur *et al.*, 2015). AFT contamination in crops is better managed using unique biocontrol techniques (Damann Jr., 2015). The main aim of this research work was to mitigate AF contamination, by identifying and characterizing the non-toxigenic specie which would open new innovations in agriculture sector through biocontrol approach promoting sustainability and food safety.

MATERIALS AND METHODS

Survey and Sample Collection

A survey was conducted in 4 Tehsils of Multan district which included (Multan city, Shujaabad, Multan Saddar and Jalalpur Pirwala) in the region of Punjab, Pakistan from October to November 2023, to quantify the incidence of aflatoxin production over the maize fields. The disease incidence was recorded by following formula.

$$\text{Disease Incidence \%} = \frac{\text{Number of infected cobs}}{\text{Total Number of observed cobs}} \times 100$$

The purpose of the study was to collect extensive data regarding the incidence of aflatoxin producing *Aspergillus* species in various Tehsils and villages of the district and to identify the non-aflatoxin producing *Aspergillus* species in cereal crops. The samples were collected randomly from 100 fields, 5 villages per Tehsil covering five fields in each village. The samples were packed in zipper bags and transported to the Diagnostic Laboratory of MNS University of Agriculture Multan and stored at 4°C for the purpose of isolating and identifying *Aspergillus* species (Mamo *et al.*, 2018).

Isolation of *Aspergillus species*

Potato Dextrose Agar (PDA) was used for isolation, purification and culturing of the *Aspergillus* species growth. 200 grams of peeled potatoes were roughly diced and mixed with 1000 milliliters of distilled water in a saucepan to make potato starch. After that, the mixture was brought in a boil till the potatoes were softened around 15 minutes. The filtered potato extract was measured carefully for making a proper concentration and poured in a glass media bottle with the addition of 20 gram of dextrose and 20 grams of agar-agar. An antibiotic (streptomycin) was added to inhibit the bacterial growth in the media according to the concentration. The suspension was autoclaved at 121°C for 15 minutes to ensure the removal of any potential impurities. The media after autoclaving was cooled and poured into sterilized 9 cm diameter petri plates in the exposure of UV light for 15-20 minutes to avoid contamination. The infected maize seeds were separated from the cobs and the seeds were disinfected with 0.5% of (NaOCl) suspension for two minutes and 3-4 changes of wash was given with distilled. The infected seeds were isolated onto the pre-filled petri plates of PDA with the help of sterilized forceps under the laminar airflow. The plates were wrapped and incubated for 2-3 days at 28°C for fungal mycelial growth (Al-Masoodi *et al.*, 2023).

Purification of *Aspergillus* species

The incubated petri plates were observed with fungal mycelial growth after 48-72 hours of incubation and the purification was done from 7 days old cultures with sterilized inoculating needle through single spore technique onto the slants containing PDA media. The plates were wrapped and incubated at 28°C for seven days. The purified cultures of *Aspergillus* species were observed after 7 days by covering the whole petri plates (Compaore *et al.*, 2021).

Morphological Identification of Aflatoxin And Non-Aflatoxin Producing Aspergillus Species

The *Aspergillus* species were identified according to their physical properties, growth, color and texture by observing the obverse and reverse side of the isolates.

Preparation of Growth Media

A number of growth media comprising Coconut Milk Agar (300gm, peptone 10 gm, yeast extract 10 gm, agar-agar 15 gm/1000 ml of distilled water), *Aspergillus flavus* and *Aspergillus prasiticus* Agar (AFPA) differentiated media (peeled potato 200 gm, dextrose 20 gm, yeast extract 20 gm and agar-agar 15 gm/1000 ml of distilled water) and PDA (peeled potato 200 gm, dextrose 20 gm, agar-agar 20 gm/1000 ml of distilled water),was prepared and autoclaved at 121°C at 15 psi for 15 minutes. The sterilized media was let to be cooled and then poured in 20 sterilized petri plates and 1 control for each of the growth media. The plates were wrapped and stored at 4°C until inoculation (Khan *et al.*, 2020).

Inoculation of Aspergillus species on Coconut Milk Agar Media

The spore suspension of 10µl from each isolate with 0.1mg of chloramphenicol was placed at the center of 20 petri plates from the 7 days old *Aspergillus* cultures, which were shifted in distilled water with 0.025% tween 80 and 1 control with 10µl of only distilled water.The petri plates were wrapped with a plastic tape and incubated at 28°C for 7 days. Then the petri plates were tested for aflatoxin fluorescence under the exposure of UV light at (365 nm) and the result was recorded (Fente *et al.*, 2001).

Inoculation of Aspergillus species on Differentiated Media

The 7 days old cultures from the incubator were unwrapped in order to inoculate the fungus on AFPA media. The single hyphal tip method was used, the sterilized inoculating needle was hot flamed and cooled, and with the help of inoculating needle single hyphal piece of 4mm disc was transferred on 20 AFPA media pre-filled

petri pates and 1 control with no inoculation. The plates were later wrapped with plastic tape and incubated at 28°C for seven days and the result was observed (Reis *et al.*, 2014).

Detection Based on Ammonia Vapor Test

The PDA media was used to detect the isolates of *Aspergillus* species using Ammonia Vapor Test (AVT). The 7 days old cultures were brought in the laminar flow chamber, the petri plates were unwrapped and the sterilized inoculating needle was hot flamed and cooled. The single hyphal tip technique was employed, a single 4mm hyphal piece was cut and transferred onto 20 PDA media pre-filled petri plates and 1 control with no inoculation. The petri plates after inoculation were wrapped with plastic tape and incubated at 28 °C for 7 days. After 7 days of incubation, the petri plates were unwrapped and a drop or two of concentrated ammonium hydroxide solution was added to all 20 petri plates at the center and the plates were again wrapped to avoid contamination. The petri plates were let for overnight and each petri plate was observed upside-down for the test and the result was observed.

RESULTS

The average of disease incidence values from the 4 Tehsils were calculated by taking the mean of disease incidence. According to the investigated data, there was significant variation in the incidence of aflatoxin producing *Aspergillus* species among the surveyed Tehsils. The result showed different levels of aflatoxin production, Tehsil Multan indicated the highest disease incidence of 26.21%. The records of the investigation showed a significant prevalence of aflatoxin by specifying a potential area of disease occurrence within Tehsil Multan. Meanwhile Tehsil Multan Saddar with a lowest disease incidence of 13.45%, Tehsil Jalalpur Pirwal 21.68%, Tehsil Shujaabad 16.10% and the average disease incidence of all 4 Tehsil was recorded as 19.36% as shown in (Figure 1). These findings presented various diversity of aflatoxin across the surveyed regions.

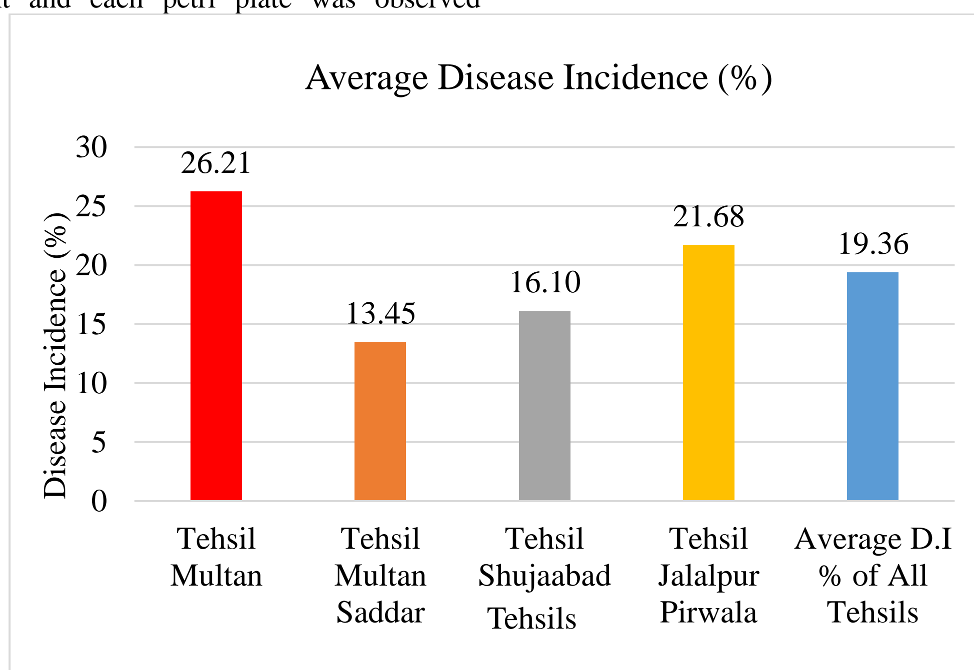

Figure 1. Average disease incidence of all four Tehsils of Multan district.

Isolation and Purification of Aspergillus species

The infected cobs collected during survey, were isolated on PDA media in the Diagnostic Laboratory at MNS University of Agriculture, Multan. The collected samples showed typical symptoms of aflatoxin. During the continuation of the investigations, the fungi were purified. In purification of the isolates two *Aspergillus* species were identified as *Aspergillus flavus* and *Aspergillus niger* based on their morphology as shown in (Figure 2).

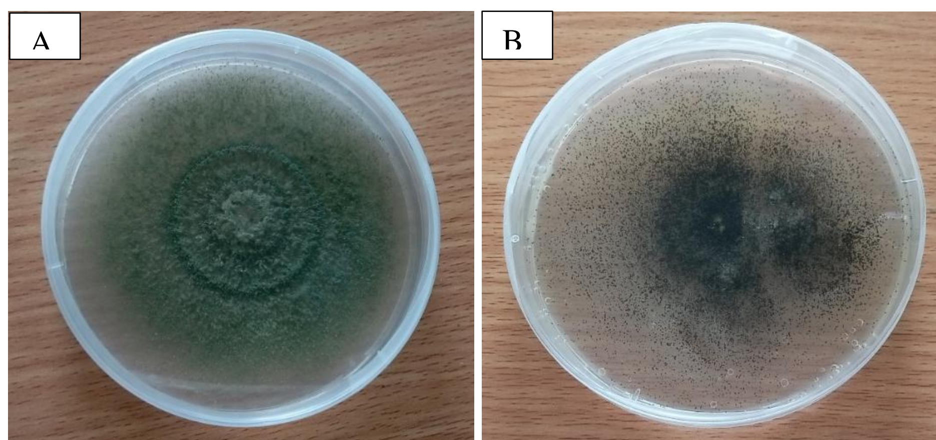

Figure 2. Pure cultures of *A. flavus* **(A)** and Pure culture of *A. niger* **(B)**.

Morphological Identification of *Aspergillus flavus* and *Aspergillus niger*

The physical properties of the colony were examined and the additional microscopic examination was conducted. The isolates were identified as *Aspergillus flavus* based on their morphology with colony color, growth and pigmentation. Initially, the mycelia appeared cottony white, later produced olive green conidia after 3 days. *Aspergillus niger* was confirmed with velvety appearance, transitioning from white to dark on PDA media. Microscopic analysis revealed distinct conidiophores and conidia structures for both species using digital and compound microscopes as shown in (Figure 3).

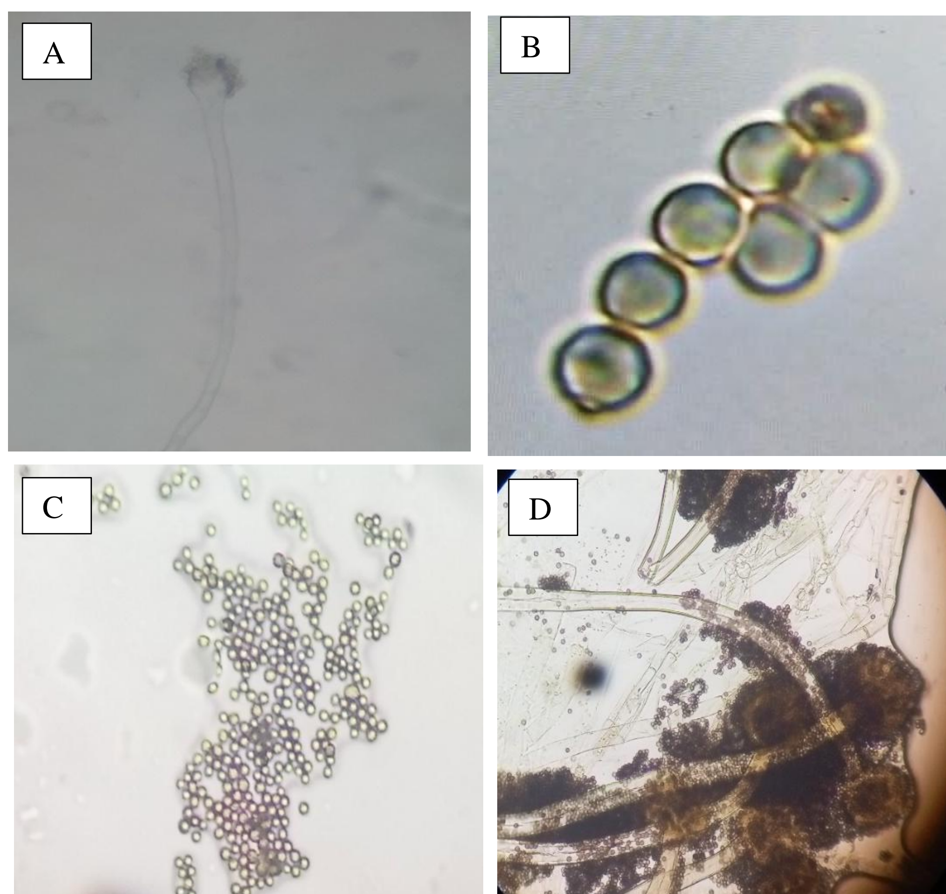

Figure 3. Digital microscopy of *Aspergillus flavus* conidia **(A)** and Conidiophore in **(B)** and Conidia of *Aspergillus niger* in **(C)** and Conidiophores in **(D)**.

Morphological Identification of Aflatoxin and Non-aflatoxin Producing Aspergillus species

Inoculation of Aspergillus species on Coconut Milk Agar Media

Aspergillus species were screened using Coconut Milk Agar (CMA) to distinguish between aflatoxin and non-aflatoxin producing isolates. The cultured isolates were exposed to UV light on a transilluminator at 365 nm. The isolates of *Aspergillus* species were displayed upside-down in UV light screening for fluorescence. Out of 20 isolate, 19 fluoresced blue which were considered as aflatoxins producing *Aspergillus flavus* isolates while 1 isolate did not exhibit blue fluorescence in UV light at 365 nm and proved to be as non aflatoxigenic isolate as presented in (Figure 4). The controlled plate didn't exhibit any color.

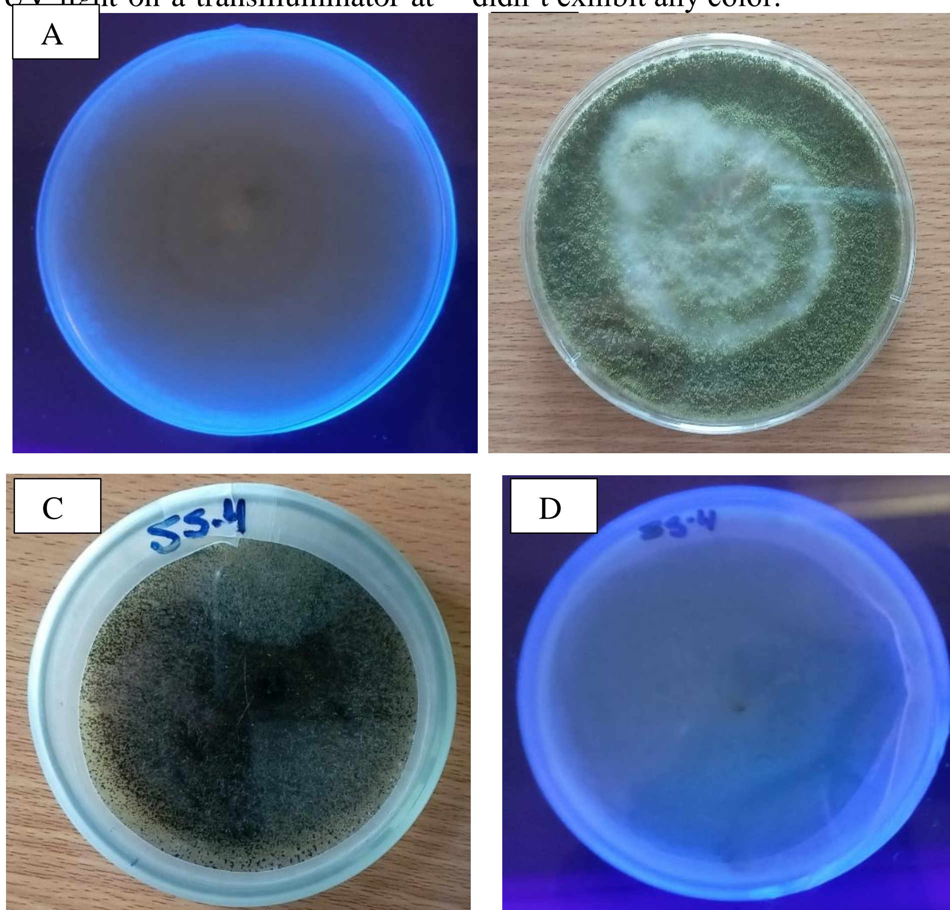

Figure 4. Obverse colony morphology of *A. flavus* on Coconut Milk Agar (CMA) media **(A)** Reverse screening of *A. flavus* under UV light exposure at 365 nm **(B).** Obverse colony morphology of *A. niger* on Coconut Milk Agar (CMA) media **(C)** Reverse screening of *A. niger* under UV light exposure at 365 nm **(D)**.

Inoculation of *Aspergillus* species on Differentiated Media

AFPA media was used for the detection of aflatoxin and non-aflatoxin *Aspergillus* isolates. All of the 20 isolates were inoculated on this media along controlled plate with no inoculation and incubated at 28°C. Aflatoxin producing *Aspergillus* species were distinguished from other species by observing an orange color underside of its colonies. Total 19 isolates produced orange color underside their colonies which were considered as aflatoxin isolates and 01 isolate did not show any orange color underside of its colony which was considered as non-aflatoxin producing *Aspergillus* isolate as presented in (Figure 5), while the controlled plated didn't show any coloration.

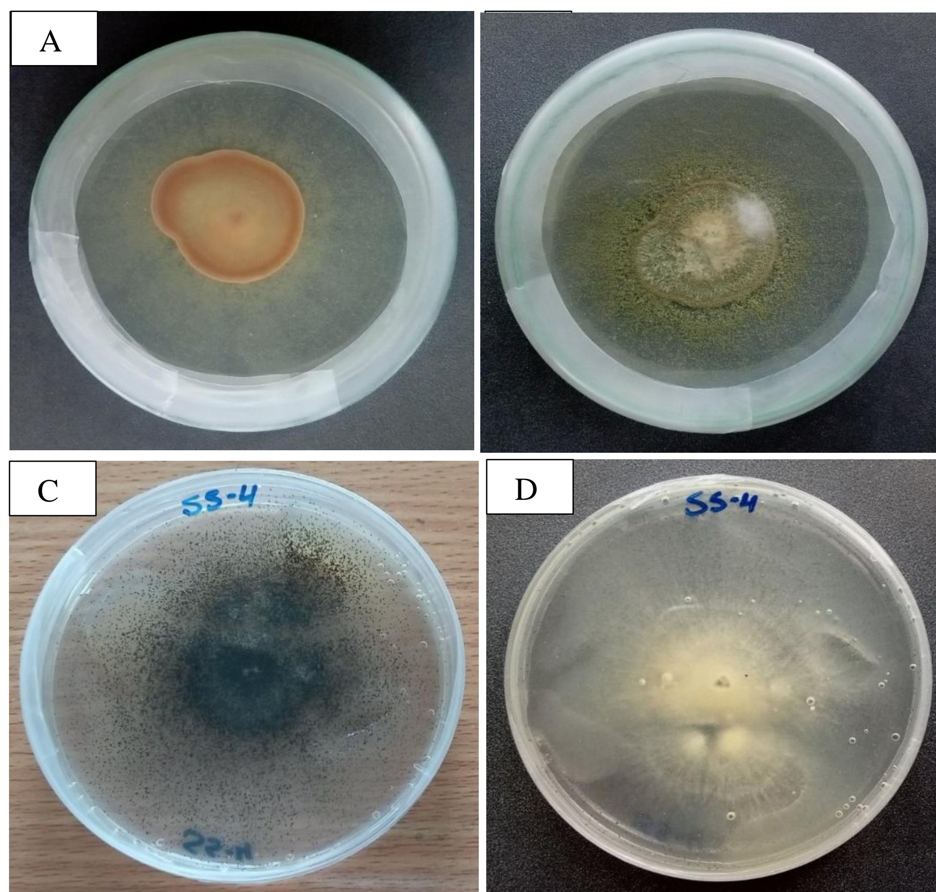

Figure 5. Obverse colony morphology of *A. flavus* on AFPA media **(A)** Reverse examination of *A. flavus* on AFPA media with orange color **(B)** Obverse colony morphology of *A. niger* on AFPA media **(C)** Reverse examination of *A. niger* on AFPA media **(D)**.

Detection Based on Ammonia Vapor Test

The isolates were detected through Ammonia Vapor Test (AVT) on Potato Dextrose Agar. On the 7 days old 20 isolates, a drop or two of concentrated ammonia hydroxide solution was added and the underside colonies were examined after 24 hours. Total 19 isolates generated plum red which were aflatoxin producing *Aspergillus flavus* species and 01 isolate did not change its underside color which was considered as non-aflatoxin generating *Aspergillus* specie as shown in Figure 6, while the controlled plated didn't show any coloration.

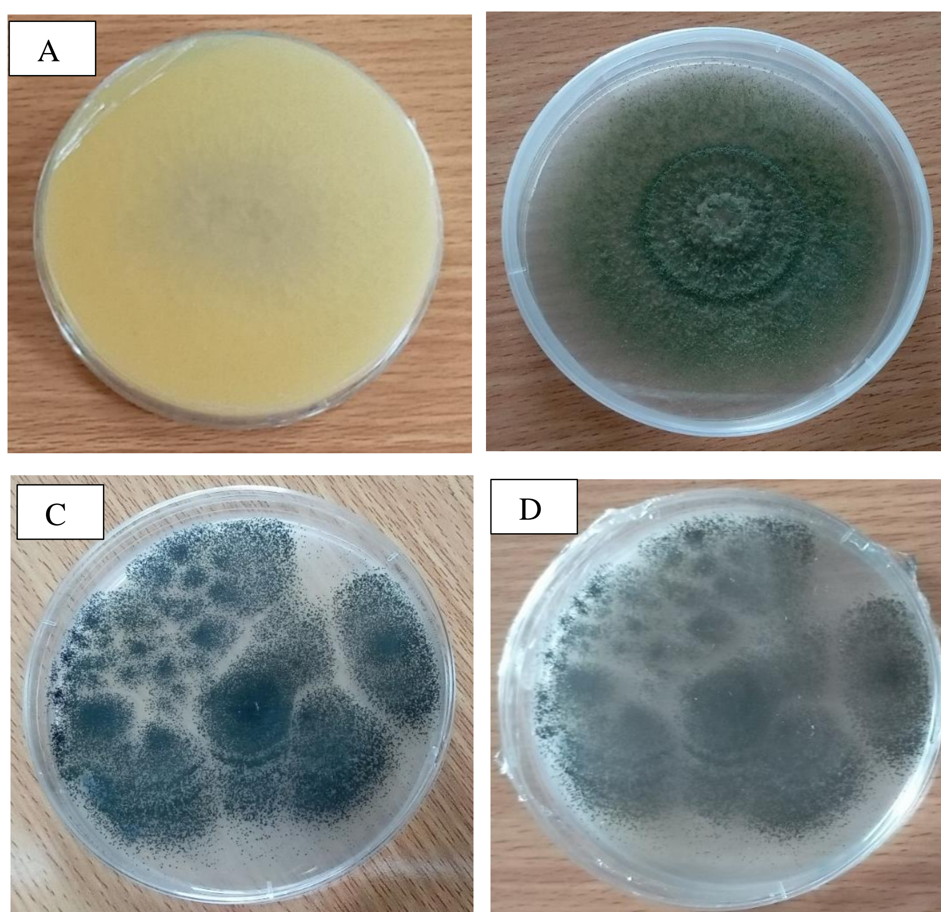

Figure 6. Obverse colony morphology of *A. flavus* on PDA media **(A)** Reverse examination of *A. flavus* on PDA after ammonia vapor test **(B)** Obverse colony morphology of *A. niger* on PDA media **(C)** Reverse examination of *A. niger* after ammonia vapor test **(D)**.

Table 1. CMA, AFPA and Ammonia vapor based detection of aflatoxin and non-aflatoxin producing *Aspergillus* isolates.

S no.	Isolates	Species	CMA Media	AFPA Media	Ammonia Vapor Test
01	MC1	*Aspergillus flavus*	+	+	+
02	MC2	*A. flavus*	+	+	+
03	MC3	*A. flavus*	+	+	+
04	MC4	*A. flavus*	+	+	+
05	MC5	*A. flavus*	+	+	+
06	MS1	*A. flavus*	+	+	+
07	MS2	*A. flavus*	+	+	+
08	MS3	*A. flavus*	+	+	+
09	MS4	*A. flavus*	+	+	+
10	MS5	*A. flavus*	+	+	+
11	SS1	*A. flavus*	+	+	+
12	SS2	*A. flavus*	+	+	+

13	SS3	*A. flavus*	+	+	+
14	SS4	*A. niger*	-	-	-
15	SS5	*A. flavus*	+	+	+
16	JP1	*A. flavus*	+	+	+
17	JP2	*A. flavus*	+	+	+
18	JP3	*A. flavus*	+	+	+
19	JP4	*A. flavus*	+	+	+
20	JP5	*A. flavus*	+	+	+
21	C1, C2, C3 Control	No Inoculation	-	-	-

DISCUSSION

Aflatoxin contamination in cereal crops has been a concern in various regions of Pakistan including Multan (Ajmal *et al.*, 2022). The climate of Pakistan which is warm and humid, is conducive to the growth of fungus, *Aspergillus flavus* is recognized as one the most significant fungi that damages corn both in the field and during storage and may experience various infection rates. Aflatoxin contamination is a major risk to the economy and food safety; management measures for aflatoxin in crops have been developed (Ashraf *et al.*, 2023). In this study, 100 fields were surveyed in Multan district, 5 villages per Tehsil and the samples were collected. The highest occurrence of *A. flavus* (26.21%) was recorded in Tehsil Multan while lowest (13.45%) incidence was found in Tehsil Multan Saddar. The variations in the environmental conditions among the fields may be the cause of differences in *Aspergillus* species occurrence. Crops that are grown in hot, humid conditions are more vulnerable to *A. flavus* and increased temperature and moisture provide suitable environment for fungal growth leading to aflatoxins contamination (Bandyopadhyay *et al*., 2016). The morphological characterization of fungi, focusing on macro and micromorphological features, is a common method for fungal identification (Pitt and Hocking, 2009). In this study, morphological characterization emphasized the importance of basic identification methods for rapid screening, especially when the access to advance tools is a great challenge. The key traits of *Aspergillus* species such as colony color, texture, sclerotia and pigmentation were observed on three different media. *A. flavus* initially appeared as cottony white and later developed olive green conidia while *A. niger* was identified by its velvety texture, transitioning from white to dark on PDA media (Thathana *et al.*, 2017). The use of CMA, AFPA and PDA media in this study enabled sufficient growth, sporulation and pigmentation, allowing for a thorough identification of aflatoxin and non-aflatoxin producing *Aspergillus* isolates. Recent literature also highlights the frequent use of these selective media for identification. The fluorescence characteristics of *A. flavus* and *A. niger* isolates under UVlight at 365 nm were screened, 19 out of 20 isolates showed blue fluorescence while non-aflatoxigenic *A. niger* showed none (Figure 4). In this study, CMA media made it possible for the rapid distinction between aflatoxigenic and non-aflatoxigenic isolates. AFPA and PDA media were also used to detect the aflatoxin and non-aflatoxin *Aspergillus* isolates. On AFPA media, 19 out of 20 isolates showed orange coloration underside their colonies which were aflatoxin producing *Aspergillus* isolates and 01 did not show any pigmentation and considered as non-aflatoxin *Aspergillus* isolate. On PDA media the isolates were detected through Ammonia Vapor Test (AVT) with a drop of concentrated ammonia hydroxide solution on 7 days old 20 isolates. The test confirmed the result, as 19 isolates turned plum red indicating aflatoxin producing isolates while 01 isolte showed no color change and confirmed as non-aflatoxin producing *Aspergillus* isolate (Table 1). The identification and characterization from Figure 4-6 are similar to that of the *Aspergillus* characteristics described by Khan et al. (Khan *et al.*, 2020). These culture-based techniques are relatively simple and cost-effective, they are typically used in combination with chromatographic methods for aflatoxin detection (Frisvad *et al*., 2019).

CONCLUSION

In conclusion, aflatoxin contamination poses a significant threat to cereal crops in Pakistan, particularly in the regions with warm and humid climates like Multan. The samples collected in the regions, included both aflatoxin and non-aflatoxin

producing *Aspergillus* species. This study highlighted the importance of distinguishing between aflatoxin and non-aflatoxin producing fungi using morphological and media-based techniques, especially when advance tools were inaccessible. The use of selective media such as CMA, AFPA and PDA proved effective in distinguishing aflatoxigenic and non-aflatoxigenic isolates, with fluorescence screening and the Ammonia Vapor Test (AVT) further confirming these findings. Integrating non-aflatoxigenic strain of *Aspergillus niger* into crop management strategies, particularly in the environment vulnerable to *A. flavus*, the risk of aflatoxin contamination could be effectively mitigated because the non-aflatoxin producing *Aspergillus* species are reported to outperform the aflatoxin producing *Aspergillus* species.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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