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Research Article

Morphological Characterization and Toxigenicity Screening of *Aspergillus flavus* isolated from Maize in Pakistan

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ABSTRACT

Aflatoxin contamination of maize caused by *Aspergillus flavus* is a serious concern due to its negative impacts on human and animal health, food security and trade. This study aims to characterize *Aspergillus flavus* isolates from maize samples collected from different locations in Punjab on the basis of morphology. Screening of toxigenic and atoxigenic isolates was also the objective of this study. Maize samples were cultured on potato dextrose agar (PDA) to get pure cultures. These were sub-cultured onto four differential media to identify the isolates using macro- and micro-morphological characteristics. Differential media included were Malt Extract Agar (MEA), Czapek Dox Agar (CZA), modified Rose Bengal Agar (RBA), and *Aspergillus flavus parasiticus* Agar (AFPA). These specialized media enable species identification based on macro-morphological characteristics. The isolates were then cultivated on Coconut Agar Media (CAM) and Yeast Extract Sucrose Media (YES) to determine their aflatoxin-producing potential based on ammonia vapor test focusing on reverse colony color changes. This study provided valuable information on the prevalence of aflatoxin-producing *A. flavus* isolates in maize in Sahiwal, Okara, Pakpattan, and Toba Tek Singh districts of Punjab, Pakistan. The study contributes to developing early detection of the aflatoxin contamination. Our research aims to maintain the economic value of this important staple crop while substantially contributing to improving food safety and security in Pakistan.

Keywords: *Aspergillus flavus*, aflatoxins, morphology, aflatoxin profiling, maize.

INTRODUCTION

Maize (*Zea mays* L.) is the third most important crop and one of the most important staple crops for billions of people worldwide (Asghar *et al.*, 2020). The contribution of maize to Pakistan's GDP is 0.7% and 3.0% of the value added in agriculture (Pakistan's Finance Division, 2022-23). In Punjab, a total of 1,720 thousand hectares of maize were planted in 2022-2023, which is 4.1 percent more than the 1,653 thousand hectares sown the year before. But it produced 10.183 million metric tons, a 6.9 percent increase over the 9.525 million metric tons it produced the previous year. The increase in production was mainly due to expansion in the cultivation area and good harvest (Pakistan Economic Survey, 2022-23). Maize is a crucial component in the production of animal feed, human food, and biofuel (El-Sabagh *et al.*, 2018). Maize is vulnerable to various fungal pathogens in the field as well as during storage conditions (Giorni *et al.*, 2019). There are several prominent fungal species such as *A. flavus*, *Fusarium proliferatum, Fusarium verticillioides, and Fusarium graminearum* that can contaminate maize grains by producing harmful toxins such as deoxynivalenol, zearalenone, fumonisins, and aflatoxins (Sun *et al.*, 2017). Aflatoxin contamination is one of the significant issues related to maize in Pakistan that affects crop quality and safety. Aflatoxin contamination in maize is caused by both pre-harvest and post-harvest factors. A wide range of food crops affected by aflatoxin contamination include cereals (like rice, sorghum, wheat, barley, and maize), oilseeds (like groundnuts, cottonseed, khopra,

Article History

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sunflower seeds, pistachios, and other nuts), and tuber crops (like cassava and yam chips) (Abdallah *et al.*, 2020). The four most significant forms of naturally occurring aflatoxins (AFs) found in a variety of crops, foods, feeds, and agricultural products are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) (ul Hassan *et al.*, 2020). AFs are mycotoxins that develop in the crop from many other types of fungi, such as *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria*, which can synthesize aflatoxins; however, the most toxic strains of aflatoxins are those identified from *Aspergillus flavus* and *Aspergillus parasiticus* (Kumar *et al.*, 2021). When exposed to aflatoxin levels above 1000 ppb, individuals may develop acute aflatoxicosis, which quickly manifests as liver damage, hepatitis, and, in some instances, death. Aflatoxin B1 was identified as the deadliest and categorized as a probable human carcinogen by the International Agency for Research on Cancer (IARC) (Okayo *et al.*, 2020). It is crucial to differentiate between toxigenic and atoxigenic *A. flavus* strains because it has been shown that only 40-50% of these strains are capable of producing toxins (Davari et al., 2015).

A. flavus is a highly virulent fungus that significantly affects maize, peanuts, and corn during both preharvest and postharvest stages. It is a saprophytic soil fungus that often produces aflatoxins, the most potent natural carcinogens, thus contaminating seed crops and damaging their quality and yield (Amaike and Keller, 2011). *A. flavus* ranks as the second most common cause of invasive aspergillosis (Rudramurthy *et al.*, 2019). Macro and micro-morphological characteristics of the fungus, together with the available taxonomic keys, have been used on differential media. Macro-morphological characteristics include the diameter of the colony growth on petri plate, colour and texture of the colony, colony colour from back side of the plate, presence of exudates, and production of fungal soluble pigments in the medium. The form and seriation of the conidia head, diameter and shape of the vesicles, the length, breadth, texture, and color of the stipe, and other micro-morphological features have all been utilized to identify *Aspergillus* species (Nyongesa *et al.*, 2015). Many secondary metabolites, including aflam, nitropropionic acid, aflatoxin B1 and aflatoxin B2, aspergillic acid, and kojic acid, are produced and released by *A. flavus* (Yu, 2004). According to the US Food and Drug Administration (USFDA), restriction must be imposed on the amount of mycotoxins permitted in food items. To ensure the safety of consumer's health, great deal of research is recommended to quantify, detect and control aflatoxin contamination (Kumar *et al.,* 2017).

A common technique for identifying aflatoxin producing *Aspergillus* species is the ammonia vapor test. Based on changes in colony color when exposed to ammonia vapors, this straightforward but efficient qualitative test offers a quick way to detect fungi that produce aflatoxins. Using this technique, just one colony will be developed in the middle of petri dish. When exposed to ammonia vapors produced by ammonium hydroxide drop placed on the petri plate lid after it has been inverted, the aflatoxigenic *Aspergillus* strain's reverse colony color turns pink, while in case of non-aflatoxigenic strain, no color is produced (Alkhersan *et al.*, 2016). This study aims to assess the prevalence of aflatoxigenic *A. flavus* isolates in maize across selected districts in Punjab, Pakistan. Our research endeavors to contribute significantly to enhancing food safety and security in Pakistan while preserving the economic value of this crucial staple crop.

MATERIALS AND METHODS

Description of the Study Site

The study was conducted with maize seed and soil samples collected from Sahiwal, Okara, Pakpattan, and Toba Tek Singh districts of Punjab, Pakistan. These districts were selected for their substantial maize production and varied soil types. Sahiwal is known for its fertile land and extensive agriculture; Okara for its mixed cropping systems; Pakpattan for its rich alluvial soil; and Toba Tek Singh for its varied agro-climatic conditions. By selecting these districts of central and south Punjab, the study aimed to capture a comprehensive range of environmental and agricultural conditions to understand better the distribution and impact of *Aspergillus flavus* and its aflatoxin production in maize.

Sample Collection

Fifteen maize seed samples were collected from the fields of above-said districts within the Punjab province of Pakistan. Four samples were obtained from Sahiwal region, six from Okara, two from Pakpattan, and three from Toba Tek Singh. Upon collection, maize seeds were stored at room temperature in the laboratory, where they were subsequently processed for isolation. Similarly, 15 soil samples from these areas were also collected. The samples were taken from the top six inches of field soil. Soil samples were collected and put in sterile plastic bags. Soil samples were stored at room temperature and were tested the next day.

Isolation and Identification of *Aspergillus flavus*

Soil and maize grain samples were utilized to isolate *Aspergillus sp*. using potato dextrose agar (PDA) media. Then morphology of isolated cultures of *Aspergillus sp*. was studied on four different media namely Malt extract agar (MEA), Czapek dox agar

(CZA), Rose Bengal Agar (RBA), Aspergillus flavus parasiticus agar (AFPA) to identify the fungus at specie level (Samson *et al.*, 2004).

Preparation of Growth Media

The growth media (PDA= 39 g/L, MEA= 50g/L, CZA= 45.5 g/L, RBA= 31.5 g/L, AFPA= 45.5 g/L) were prepared following the manufacturer's instructions and sterilized at 121°C and 15 psi pressure for 15 min. After cooling for 20 minutes, the sterilized medium was poured onto 90 mm petri dishes. It was maintained at 4°C till the inoculation.

***Aspergillus flavus* from Maize Grain Samples**

Maize grain samples were observed under a stereoscope and infected grains were processed for isolation. Selected seeds were surface sterilized by following the method used by (Karanja, 2013). Four to five maize kernels were surface sterilized by using 70% ethanol for 1-2 minutes followed by three washing with distilled autoclave water and drying on sterile filter paper. The samples were then inoculated on PDA plates supplemented with streptomycin sulfate (130 µg mL^{-1}) to inhibit bacterial growth. Positive control plates were inoculated with *A. flavus*, while negative control plates were left uninoculated for comparison. After seven days of incubation at 28°C, the uninoculated and infected plates were examined every day for spore generation and growth. The fungal colonies were purified on newly made PDA and cultured for an additional seven days after the initial seven days period. We used an InnoTECH stereo-binocular microscope to observe colonies of *A. flavus*. After purification, representative *A. flavus* isolates were cultivated on different plates.

***Aspergillus flavus* from Soil Samples**

To perform the soil sampling procedure, one gram soil sample was taken and thoroughly mixed with 10 mL of sterile water, ensuring a well-mixed solution for approximately 2 to 3 minutes using a rotary shaker. After making serial dilutions, the mixture was plated onto PDA at an appropriate dilution of 10^5. Positive control plates were inoculated with *A. flavus*, while negative control plates were left uninoculated for comparison. The inoculated and uninoculated plates were cultured for five days at 28°C, and their growth and spore generation were monitored every day. The fungal colonies were sub cultured on newly made PDA and incubated for an additional five days following the first five days incubation period. We used an InnoTECH stereo-binocular microscope to see colonies of *A. flavus*. To get pure culture, representative isolates of *A. flavus* were subsequently single-spored. According to Klich, 2002, isolates of *A. flavus* were identified using morphological keys and colony morphology.

Morphological Confirmation of *Aspergillus flavus*

The isolates were cultivated on AFPA and incubated for seven days at 28°C in order to verify the identity of *A. flavus* by colony reversal color.

Aflatoxin Profiling of *Aspergillus flavus* by Using Ammonia Vapor Test

Coconut milk agar (CMA) and yeast extract sucrose agar (YESA) were used to screen the production of aflatoxin among the strains. After inoculating the middle of the petri plates with CMA, YESA, and 0.1 mg of chloramphenicol with 7 days old *A. flavus* spores, the plates were cultured for seven days at 28°C in the dark. Following this incubation period, the petri dish was inverted, and 1 ml of concentrated ammonium hydroxide solution was carefully placed on the side of the lid of the 90 mm petri plate for the screening of aflatoxin. Results were observed after 20 minutes (Alkhersan *et al.*, 2016).

RESULTS

Detection of *Aspergillus flavus*

Fifty fungal isolates were isolated from maize grain and soil samples. The isolated species of fungi were identified by recording their morphological and microscopic characters. *A. flavus* culture was identified by using the key described by (Klich, 2002). A chi-square goodness-of-fit test was applied to compare the incidence of *A. flavus* across four sampled districts. The observed incidence percentages were 18% in Sahiwal, 20% in Okara, and 12% each in Pakpatan and Toba Tek Singh. The test revealed no significant variation among districts ($\chi^2(3) = 3.29$, $p > 0.05$) (Figure 1). This study aims to focus on morphology and microscopy of *A. flavus* cultures because this fungus is responsible for aflatoxin production in maize grains.

Comparison of Morphological Characteristics of *Aspergillus flavus* on Five Different Nutrient Media

The *A. flavus* colonies morphology first exhibited a white mycelial hue on PDA. After three days, the colony's appearance was dominated by olive green conidia. After seven days, the colony had a white border with an olive-green raised center. The colony texture is mostly powdery smooth on PDA and to some extent powdery rough and velvety smooth. The colony diameter ranged from 60-75mm. On PDA in most of the samples, exudate is present and soluble pigments are also present. The reverse color of the colony is pale yellow to cinnamon brown.

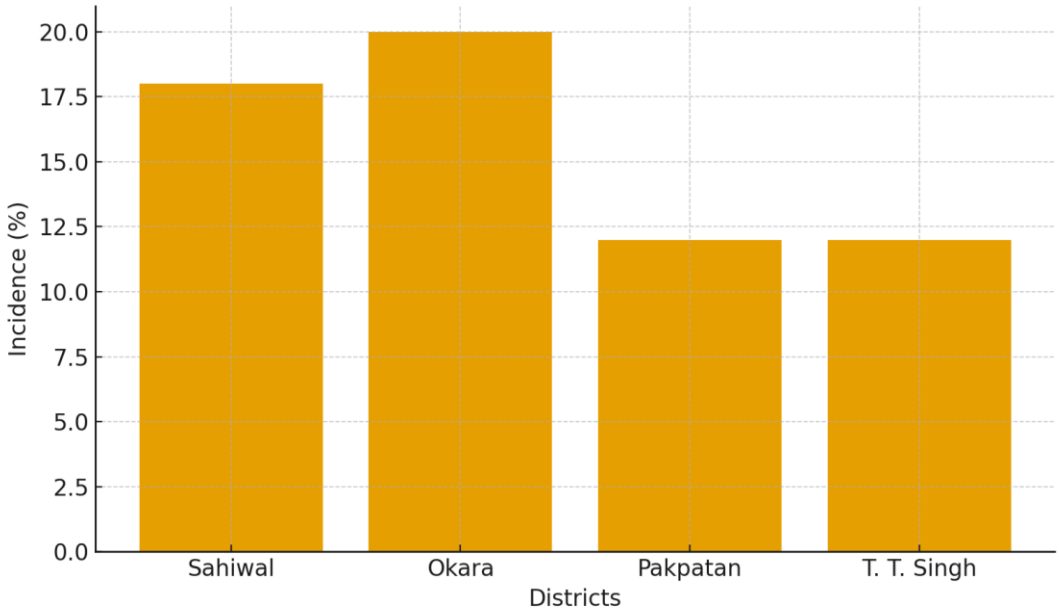

Figure 1. Incidence of *A. flavus* in different districts of Punjab. Percent incidence values were calculated based on the number of samples having *A. flavus* divided by total number of samples collected.

On MEA, *A. flavus* colonies initially produced white color. After 72 hours, the colonies turned to bright yellow and finally attained the olive-green color. The colony texture mostly appeared powdery and rough. The colony diameter ranged from 45-60 mm. Exudates were present in most of the samples and there were no produced soluble pigments. The reverse color of the colony varies from colorless to pale yellow.

Similarly, on CZA the colony growth started appearing white after 24 hours and then turned olive green to dark green with the passage of time. The colony texture was mostly floccose. The colony diameter ranged from 45-60 mm. Exudates are present in only a few samples, while there is no production of soluble pigments and no reverse colony color.

Inoculation of *A. flavus* on RBA resulted in colony growth similar to MEA media that initially produced white colony growth. After 72 hours, colonies turned to bright yellow, scattered and finally attained the olive-green color. The colony diameter ranged from 60-75 mm. Exudates are present in most of the samples and there are no produced soluble pigments and no reverse colony color.

The colony growth of *A. flavus* on AFPA appeared white after 24 hours and then remained white even after 7 days. The colony texture appears fluffy and the colony diameter ranged from 60-75 mm just as in the case of PDA and RBA. Exudates are not present, while there is no production of soluble pigment. The reverse color of the colony is bright orange to dark orange. Additionally, the comparison of morphological characteristics on five different media is summarized in table 1.

Table 1. Morphological characteristics of *Aspergillus flavus* on five different nutrient media

PDA	MEA	CZA	RBA	AFPA
Colony growth Colonies initially developed the white color of mycelia. After three days, the colony produced olive-green conidia, dominating the colony's appearance. After 7 days the colony had white board with an olive-green color and raised center. **Colony texture**: Powdery to velvety **Colony diameter** 60-75mm **Presences of exudate** In most of the samples, exudate is present **Presences of pigments** Pigment is present in most of the samples. **Reverse colony color** Pale yellow to cinnamon brown.	**Colony growth** Initially produced white. After 72 hours returned a bright yellow. Finally attained olive green color. **Colony texture** Mostly velvety and rough. **Colony diameter** 45-60mm **Presences of exudate** In most of the samples, exudate is present. **Presences of pigments** Pigments were not produced in most of the samples. **Reverse colony color** Varies colorless to pale yellow.	**Colony growth** Starts appearing after 24 hours white and then turned olive green to dark green. **Colony texture** Mostly floccose. **Colony diameter** 45-60mm **Presences of exudate** In most of the samples, exudate is not present **Presences of pigments** Pigments were not produced in most of the samples. **Reverse colony color** Not produced color.	**Colony growth** Initially produced white. After 72 hours turned bright yellow and scattered. Finally attained the olive-green color **Colony texture** Mostly smooth and rough **Colony diameter** 60-75mm **Presences of exudate** In most of the samples, exudate is present. **Presences of pigments** Pigments were not produced in most of the samples. **Reverse colony color** Not produced color.	**Colony growth** Initially produced white. It remained white even after 7 days. **Colony texture**: White fluffy **Colony diameter** 60-75mm **Presences of exudate** In most of the samples, exudate is not present. **Presences of pigments** In most of the samples, pigments were not produced. **Reverse colony color** Bright orange to dark orange color.

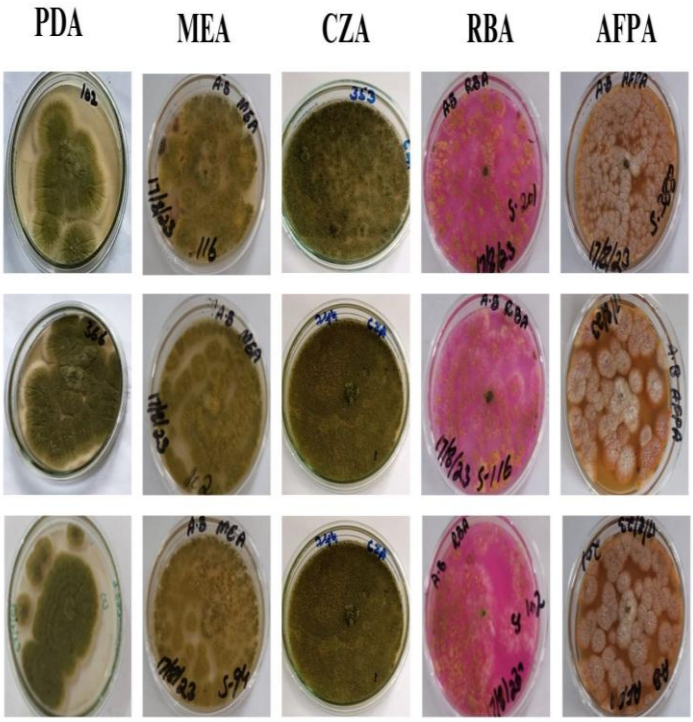

Figure 2. Petri plates showing colony morphology of *A. flavus* on five different media. The plates were incubated at 28°C. Pictures were taken seven days post inoculation (dpi) using a mobile camera (16 megapixels).

Microscopic Characteristics of *Aspergillus flavus*

The microscopic features of *A. flavus* are illustrated in Figure 3. When observed under the microscope, *A. flavus* conidiophores appeared colorless, possessing thick walls, a rough texture, and bearing vesicles. The shape of the conidia head of *A. flavus* samples is mostly globose but in some samples are sub-globose. Cells were observed to be either uniseriate or biseriate, with phialides growing on metulae. Metulae enveloped the surface of the vesicles and were dispersed in all directions. The vesicles were predominantly globose to sub-globose in shape, with diameters ranging from 18-40 µm. The strip lengths ranged from 400-800 µm, strip widths ranged from 7-14 µm, and a slightly rough texture and colorless strip color. Conidia appeared globose, possessing thin walls smooth, a slightly rough texture, and diameters ranging between 3-5 µm. These measurements were made by using micrometer scale adjustment in microscope.

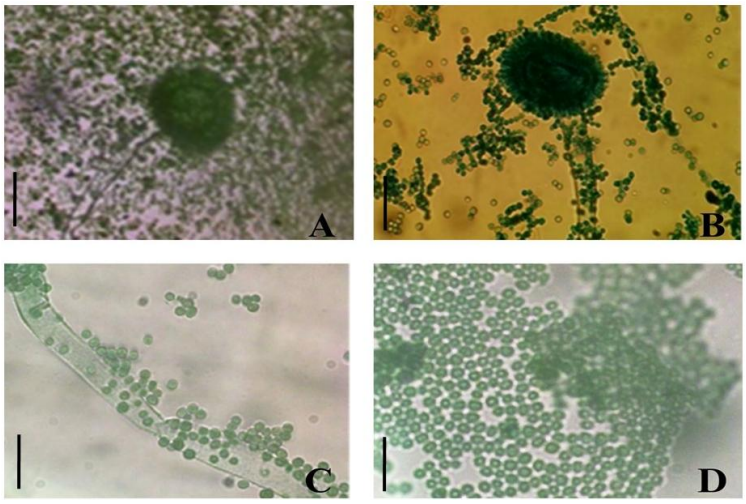

Figure 3. Microscopic characteristics of *A. flavus*. The pictures were taken at 40 X magnifications. **(A)** Uniseriate cell under 40X **(B)** Biseriate cells **(C)** Conidiophores aggregation **(D)** Conidia dispersed around conidiophore head. Scale Bar = 10 µm.

Screening of Aflatoxigenic and Non-Aflatoxigenic *Aspergillus flavus* Based on Ammonia Vapor Test

Yeast extract sucrose agar (YESA) and coconut milk agar (CMA) are commonly utilized media for screening of aflatoxin-producing (toxigenic) and non-producing (atoxigenic) *A. flavus* isolates. To characterize toxigenic *A. flavus* isolates, an ammonia vapor test was employed, wherein pink and yellow-colored colonies were observed in an inverted petri dish upon applying 1 ml of concentrated ammonium hydroxide solution on the inner side of the lid. Conversely, non-aflatoxigenic isolates did not exhibit any color change. Using ammonia vapor test to detect aflatoxigenic and non-aflatoxigenic *Aspergillus flavus*, it was found that 20 isolates (66%) were positive, while 10 isolates (33%) were negative for aflatoxigenicity. The ammonia vapor test distinguished aflatoxigenic isolates by producing a pink to red and yellow color in the center of the glass petri dish of CMA and YESA in the reverse, respectively; while non-aflatoxigenic isolates did not produce any coloration (Figure 4).

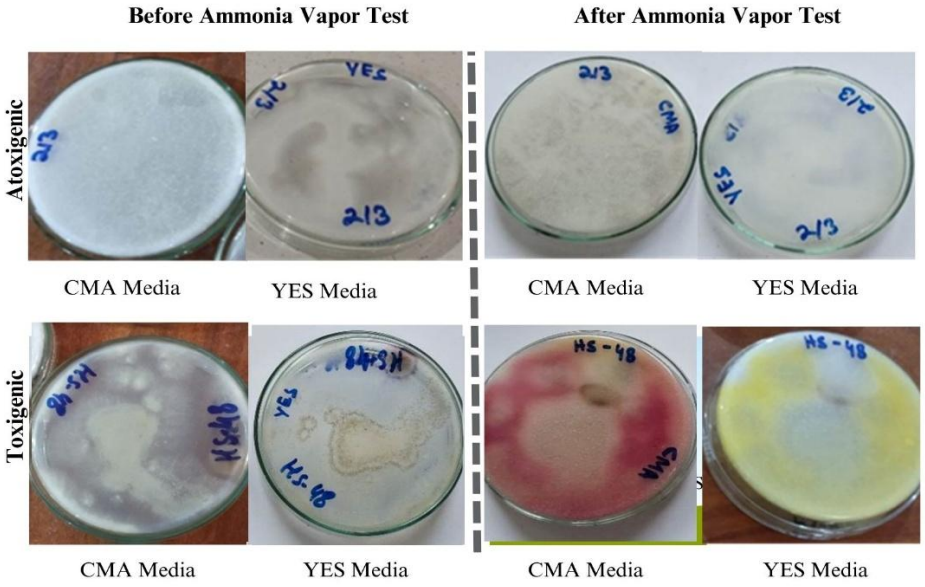

Figure 4. Detection of aflatoxigenic *A. flavus* by ammonia vapor test.

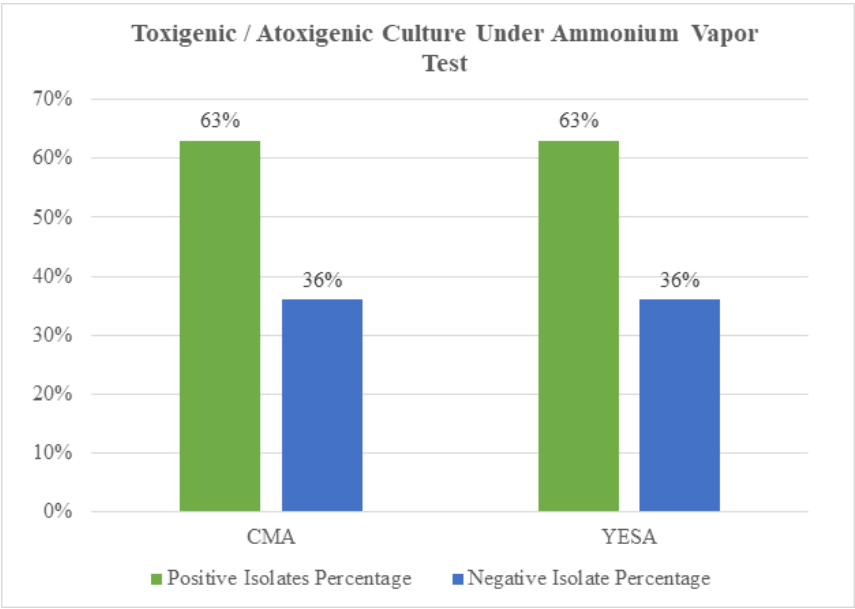

Figure 5. Graphical representation of detection of atoxigenic and toxigenic *A. flavus* isolates on CMA and YESA media.

DISCUSSION

Aflatoxin contamination in maize is a serious global issue due to its mutagenic and carcinogenic effects. It offers grave concern to both human and animal health, particularly in regions with tropical climates and recurrent drought, which favor mold growth. Aflatoxins, produced by *A. flavus* can contaminate maize before and after harvest, leading to compromised food security and economic losses from trade restrictions. The present investigation demonstrated the prevalence of *A. flavus* in seed and soil samples of maize obtained from Punjab, Pakistan. In this study, 30 isolates of *A. flavus* were identified based on morphology and microscopy along with other fungal pathogens isolated from maize grain and soil samples of Sahiwal, Okara, Pakpattan, and Toba Tek Singh districts in Punjab, Pakistan. According to the results, highest (20%) incidence of *A. flavus* was found in maize grains and soil of Okara while the lowest (12%) was recorded for Pakpattan and Toba Tek Singh districts. On the other hand, Sahiwal area had an 18% incidence of *A. flavus*. Though, these districts are not very far from each other and lie within a diameter of 140 km, however, microclimates of these areas vary to some extent. Perhaps, that is the reason for the differences in *A. flavus* occurrence in certain areas. *A. flavus* primarily infects crops produced in hot climates, while in certain places, contamination may occur as soon as the temperature increases with high humidity (Thathana *et al*., 2017).

Mycological exams (morphology-based) for the detection of microbiological pathogens were recorded in the survey of the American Society for Microbiology (ASM) (Diba *et al*., 2019). In this study, macroscopic characteristics of *A. flavus* exhibiting fast growth on PDA media align with the findings of (Afzal *et al*., 2015) who did similar work and detailed the fast growth of *A. flavus* on PDA medium. According to (Thathana *et al.*, 2017), the most significant growth rate of *A. flavus* was observed on PDA, MEA, and RBA with colony diameters ranging from 65-75 mm, 55-75 mm, and 50-70 mm, respectively.

One widely used technique for fungal identification is the macro morphological properties of fungi grown on various media, which are seen during morphological characterization. Using these morphological properties, it was possible to reliably identify *A. flavus* isolates. *A. flavus* was examined using five distinct culture media and its macroscopic features were noted, which include the colony's color, texture, and edges, as well as its exudates and soluble pigmentations. The colonies of *A. flavus* displayed colors ranging from olive green to dark green, often accompanied by a white ring that later became obscured by the formation of conidia. Following taxonomic classification parameters interpreted by Klich, 2002, the isolates in this study have colony morphology comparable to that of *A. flavus*. On PDA, *A. flavus* colonies with a granular texture and a yellow-olive green color have also been noted. *A. flavus* colonies on the PDA media surface may have light to cinnamon-brown spores under the colony and yellow-green spores on the colony's upper side (Hasanin *et al*., 2019).

However, it might be challenging to distinguish some other *Aspergillus* species from *A. flavus* since they have similar behavioral traits. It is recognized that *A. parasiticus* has the same colony color. This implies that both microscopic and macroscopic characteristics must be carefully examined in order to properly identify and categorize. Nevertheless, even when examined under a microscope, it is generally accepted that Aspergillus isolates share several characteristics (Cotty and Jaime-Garcia, 2007). In this study, which is in accordance with (Nyongesa *et al*., 2015), microscopic characteristics of *A. flavus* were noted, such as conidiophores, vesicle shape, vesicle diameter, seriation (uniseriate/biseriate), and conidia (form, size, texture, color, and wall thickness).

A. flavus was found to have uniseriate and biseriate cells with radiating vesicles under microscope. These findings are consistent with those of El-Kadi et al., 2018, who also found isolates of *A. flavus* on PDA medium. They discovered that *A. flavus* possesses colorless conidiophores and radiating conidial heads. Similarly, Thathana et al., 2017 described uniseriate and biseriate *A. flavus* cells with radiating vesicles. Chains of conidia, colorless conidiophores, and radiating conidial heads were also discovered. In addition, Afzal et al., 2013, 2015 reported comparable results of conidial chains and hyaline conidiophores allowing them to identify *A. flavus*. Teh and Latiffah, 2018 also observed the presence of globose to sub-globose vesicles containing conidia, as well as the hyaline conidial heads of *A. flavus.* However, morphological characteristics of *A. flavus* on CZA media was contrary to our results as described by Derbalah et al., 2020. On CZA agar, they reported seeing granular, flat colonies that ranged in hue from yellow-green to dark green.

A. flavus has often been identified using the particular growing media which are included in this study (Pitt et al., 1983). Similarly, Diba *et al.*, 2007 described the *Aspergillus* species from environmental and medical samples by examining their microscopic and macroscopic characteristics and reported that the use of AFPA, RBA, PDA, and other growth media may promote growth and sporulation of *Aspergillus*.

A. flavus isolates were screened on CMA and YESA medium, thus allowing for quick differentiation of toxigenic from atoxigenic isolates by qualitative testing. Thirty isolates were evaluated in this work using the ammonia vapor test, on CMA and YESA media. 20 (66%) isolates were toxigenic (positive) and 10 (33%) were atoxigenic (negative) on both CMA and YESA media. The ammonia vapor test is a common method for identifying aflatoxins in agricultural products, especially fungi like *Aspergillus*. This qualitative test detects toxigenic strains by inducing a color change in colonies when exposed to ammonia vapor. In toxigenic strains, the reverse colony turns pink, while atoxigenic strains show no color change. The majority of impoverished countries lack sophisticated technologies for detecting aflatoxin; hence, the quick screening of isolates using culture-based approaches may be helpful in these situations.

Toxigenic fungi cause severe post-harvest losses that is evident from studies like *Fusarium equiseti* in mandarins (Moosa et al., 2021). Furthermore, Ahmed et al., reported that postharvest damage imparted by toxic fungi results in poor quality fruits like figs, emphasizing the need for early detection (Ahmed et al., 2019). The toxicological risks associated with dietary contaminants have also been highlighted in studies with medicinal plants, further emphasizing the health risks posed by aflatoxins (Ahmad et al., 2020). Screening studies are frequently carried out to explore crop plants for traits including disease resistance, yield, growth etc. This practice is indeed a classical way to screen for plants with certain characters. Similarly, screening studies in crops like tomato reinforce the need to explore the maize cultivars that harbor resistance against fungal pathogens for sustainable management of the aflatoxin (Yasin et al., 2021). Hence, this study conducted on stored maize seeds reports for the presence of aflatoxin residues to toxic levels. Further, it alarms and encourages scientific community to invest in research areas like plant defense, stress adaptation, and integrated plant disease management that could reduce the risk of aflatoxin.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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