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# Isolation, diagnosis and determination of the pathogenicity of *Aspergillus* spp. from Maize seed

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**ABSTRACT. Background:** Maize plant is often induced by various types of naturally occurring pathogens such as bacteria, virus, fungi and nematodes etc. and are detrimental to the yield and quality of grains and subsequently affect the economy and threaten the food security around the globe. This study aimed to isolates the *Aspergillus* from seeds of Maize and determines their pathogenicity. **Methods:** Maize seeds were collected and cultured in appropriate media for isolation and identification of fungi from the infected seeds by morphological and microscopic features of the fungal colonies. Isolates of Aspergillus flavus were subsequently isolated and molecularly diagnosed. The ITS region was amplified using polymerase chain reaction (PCR) and DNA was extracted. Isolates so obtained were then analyzed by agar gel electrophoresis. In vitro aqueous agar pathogenicity testing was also conducted. Discs of fungal growth were placed on plates and surface-sterile maize seeds placed on the medium and an uninoculated control plate was also prepared. The plates were incubated under suitable temperature conditions, and the infection rate was observed to evaluate the pathogenicity of isolates of fungus. **Results:** The results investigated numbers of different fungal isolates from infected maize seeds. The high number of fungal isolates were *A. flavus* with 24 (48%), while the lowest isolates were *penicillium* with 3 (6%).The DNA extraction of 12 *A. flavus* isolates showed 100% positivity in agarose electrophoresis with 550 bp .The twelve isolates of *A. flavus* exhibited a variation in the infection rate of corn seeds ranging from 17.50% to 85.00% with an average of 44.18% in the result of the laboratory pathogenicity test. Isolate No. (12) had the highest infection rate (85.00%). Among the isolates, isolate 10 had the lowest infection rate of 17.50%, indicating a lesser pathogenicity than the other isolates. However, no infection was recorded in the control group with an infection rate of 0.00%. **Conclusion:** The study showed that a wide variety of fungal species is associated with maize seeds, of which Aspergillus flavus was the most common fungus. The selected *A. flavus* isolates were successfully amplified into IRS, which was confirmed by molecular analysis. The pathogenicity assay showed significant differences in pathogenicity of the fungal isolates, suggesting their differences in pathogenicity potential. The control treatment showing that no infection was present further substantiates the importance of fungal inoculation in causing seed infection.

***Keyword:*** *Maize1, Aspergillus2, Fusarium3, ITS4.*

## INTRODUCTION

Maize (*Zea mays* L.) ranks as the third most extensively farmed cereal grain globally and plays a significant role in ensuring food security worldwide. Corn may be cultivated in temperate, tropical, and subtropical regions[1]. The advancement of agriculture has rendered infectious plant illnesses a progressively critical element influencing agricultural output and economic viability[2].Maize plant is often induced by various types of naturally occurring pathogens such as bacteria, virus, fungi and nematodes etc. and are detrimental to the yield and quality of grains and subsequently affect the economy and threaten the food security around the globe [3].

Fungi are recognised for inducing degradation and nutrient depletion in maize following insect activity [4]. The fungal taxa Aspergillus, Bipolaris, Curvularia, Fusarium, and Penicillium, recognised for their seed pathogenicity, have been associated with maize seeds. Aspergillus flavus is the predominant species of Aspergillus found in the soils of West Africa and the United States[5]. Fungal infestations on maize systematically diminish its viability, nutritional quality and quantity, lead to seedling blight, hinder germination, weaken seedlings, and result in subpar crop performance [6]. Aspergillus flavus is a saprophytic pathogen that predominantly flourishes on various organic nutrient substrates containing sugar[7]. It is a fungus with significant economic repercussions that

affects animals, insects, and plants, leading to storage decay in numerous crops and the generation of the highly regulated mycotoxin, aflatoxin B. Aflatoxin impurities have been documented in certain agricultural commodities [8]. *A. flavus*, a prominent and ubiquitous fungus, is capable of enduring various environmental conditions. They exhibit a propensity to endure temperatures ranging from 12 °C to 48 °C, with an ideal growth temperature between 28 °C and 37 °C, and thrive in humidity levels exceeding 80% [9]. Maize seeds have been documented to be contaminated with *A. flavus* in agricultural settings during harvest and during storage [10]. This study aimed to isolates the *Aspergillus* from seeds of Maize and determines their pathogenicity.

## MATERIALS AND METHODS

### 2.1 Plant sample collection

Specimens of maize seed exhibiting signs of infection, such as discolouration, browning, necrosis, and wilting, were gathered between March and May 2026. Infected flora were enclosed in zipper-polyethylene bags labelled with the location, date, and type of collection zone. Subsequently, specimens were conveyed directly to the laboratory for the purpose of pathogen isolation and additional examinations.

### 2.2 Isolation of fungal plant pathogens

50 grains were taken from each sample of yellow corn kernels. They were surface-sterilized with a commercially available bleaching agent (2%) sodium hypochlorite solution for 3 minutes, washed 3 times with sterile distilled water, and dried with filter paper. The grains were cultured on potato dextrose agar (PDA) in 9 cm diameter Petri dishes, with 5 grains per dish (4 periphery and 1 in the center). The dishes were incubated for 7 days at 25 ± 2°C.

Fungi were identified according to morphological characteristics based on the approved taxonomic keys [11]. The frequency of the fungi associated with the tested samples was recorded according to the following equation:

$$\text{Percentage frequency} = \frac{Number\ of\ fungal\ isolates}{Total\ number\ of\ isolates} \times 100$$

The fungal hyphae were transferred from all Aspergillus isolates for purification using an isolation needle and cultured on PDA medium in another dish. The dishes were then incubated at 25 ± 2°C for 7 days, using the repeated culture method [12].

### 2.3 Molecular diagnosis of Aspergillus isolates

#### 2.3.1 DNA Extraction

The DNA of the Aspergillus isolate was extracted using the Bioneer kit from Korea, according to the manufacturer's instructions.

#### 2.3.2 Amplification of DNA

Sidharthan et al. [13] state that tiny tubes holding five microlitres of Master Mix were utilised. Five microlitres of extracted DNA, four microlitres of the starter (ITS4) (two microlitres forward and two microlitres reverse), and the nitrogenous base sequence. Eleven microlitres of sterile ionic distiller water were then added. In order to perform PCR amplification, which entails DNA denaturation, primer attachment to the detached strand (annealing), and DNA extension under the conditions indicated in Table (1) below, the materials were combined using a vortex device before the tubes were moved to a thermocycler:

Table 1. PCR reaction conditions

| PCR steps | Repeated cycle | Temp. (C) | Time (min) |
|---|---|---|---|
| Initial denaturation | 1 | 94 | 4 |
| Denaturation | 30 | 94 | 1 |
| Annealing | | 55 | 1 |
| Extension | | 72 | 2 |
| Final extension | 1 | 72 | 10 |

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Table 2. Primers used for ITS amplification in the polymerase chain reaction (PCR)

| Primer | Primer sequence |
|---|---|
| ITS1 (forward) | 5′-TCCGTAGGTGAACCTGCGG-3′ |
| ITS4 (reverse) | 5′-TCCTCCGCTTATTGATATGC-3′ |

**2.3.3 Gel electrophoresis**

One gram of agarose (concentration: 1%) was dissolved in ninety millilitres of sterile distilled water and ten millilitres of buffer solution (10X) to create agarose gel. After reaching the boiling point in a heat heater, the mixture was cooled to 65°C. Five microlitres of ethidium bromide were then added. After gently shaking the material to mix it, it was poured into the electrophoresis apparatus's specified area. In order to create pits in the gel, a UV-sterilized comb was added to the mixture. The mixture was then allowed to solidify for 30 to 45 minutes at room temperature. After removing the comb, the first well (which contained standard DNA fragments) was filled with five microlitres of Ladder. The remaining wells were then filled with the same volume of extracted DNA. After covering the equipment, electrophoresis was carried out at 70 volts for 60 minutes in order to identify the PCR products—that is, the amplified and extracted DNA bundles—and compare them with the standard ladder. After that, the material was transferred to Bioneer, a Korean business, for nucleotide sequence analysis.

**2.4 *In vitro* pathogenicity assessment**

A pathogenicity test was performed on the Aspergillus isolates. After the medium had solidified, 9 mm Petri dishes containing 20 g L-1 of water agar were autoclaved with amoxicillin added. The plates were then inoculated in the center with a 5 mm disc using a sterile cork puncture close to the edges of the fungal colony growing on the PDA medium at the age of seven days. Ten sweet corn seeds were then dried on sterile filter paper and dispersed in a circle after being superficially sterilised with (NaOCl) for two minutes in sterile water to eliminate any leftover solution. Each isolate, together with the control that did not contain the pathogenic fungus, was utilised in three replicates and incubated at 25±1 °C. After the uninfected control treatment had fully grown, the proportion of infection was determined. A completely randomised design (CRD) was used to assign treatments to experimental units at random. All data were subjected to analysis of variance (ANOVA) and mean differences were examined at the 0.05 level of probability ($P \leq 0.05$) based on the least significant differences (LSD).

$$Disease\ incidence = \frac{\text{No} \cdot \text{of infections}}{\text{Total No} \cdot \text{of plants}} \times 100$$

**2.5 Preparation of inoculum**

The fungal inoculum is usually propagated using millet seeds (*Panicum miliaceum* L.). 150 g of millet seeds were autoclaved for two days at 121 °C and 1.5 kg cm2 for 20 minutes after being repeatedly cleaned with tap water and left overnight in a 300 ml glass beaker. Each glass beaker containing previously autoclaved millet seeds was filled with five 5 mm diameter active growth discs from the fungal colony. The nozzle was blocked with aseptic cotton, and the mixture was thoroughly shaken to ensure homogeneity. In order to guarantee uniformity and full fungal colonisation, the inoculum was lastly cultured for 14 days at 25±2 °C with constant shaking.

**2.6 Statistical analysis**

Using a completely randomised design (CRD), the SAS Statistical Analysis System (2012) statistical software was utilised to analyse the data and examine the impact of various treatments on the attributes under investigation. The Least Significant Difference (LSD) test was then used to examine significant differences between means.

## RESULTS AND DISCUSSION

### Isolation and identification of fungal spp. from maize seed

The results investigated numbers of different fungal isolates from infected maize seeds as shown in table(3) and figure(1). The high number of fungal isolates were *A. flavus* with 24 (48%), followed by *Fusarium* with 10 (20%), *A. nigar* with 8 (16%), while the lowest isolates was *penicillium* with 3 (6%).

Table (3): number and percentage of fungal isolates from infected maize seeds

| Fungal spp. | Number (%) |
|---|---|
| *Aspergillus* spp. | 5 (10%) |
| *Aspergillus nigar* | 8 (16%) |
| *Aspergillus flavus* | 24 (48%) |
| *Fusarium* | 10 (20%) |
| *Penicillium* | 3 (6%) |
| *Total* | 50(100%) |

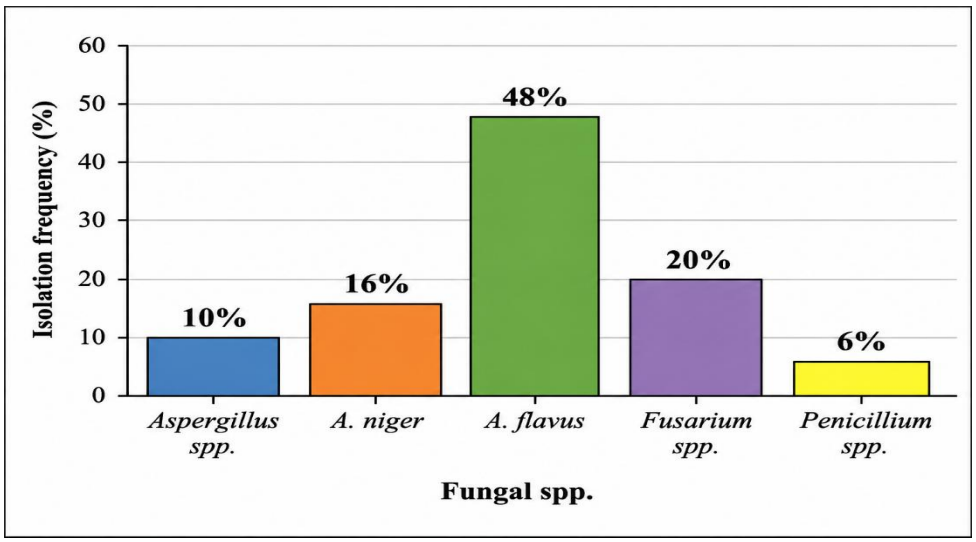



Figure 1. number and percentage of fungal isolates from infected maize seeds

### Molecular diagnosis of *Aspergillus flavus*

The DNA extraction of 12 *A. flavus* isolates showed 100% positivity in agarose electrophoresis with 550 bp as shown in figure(2).

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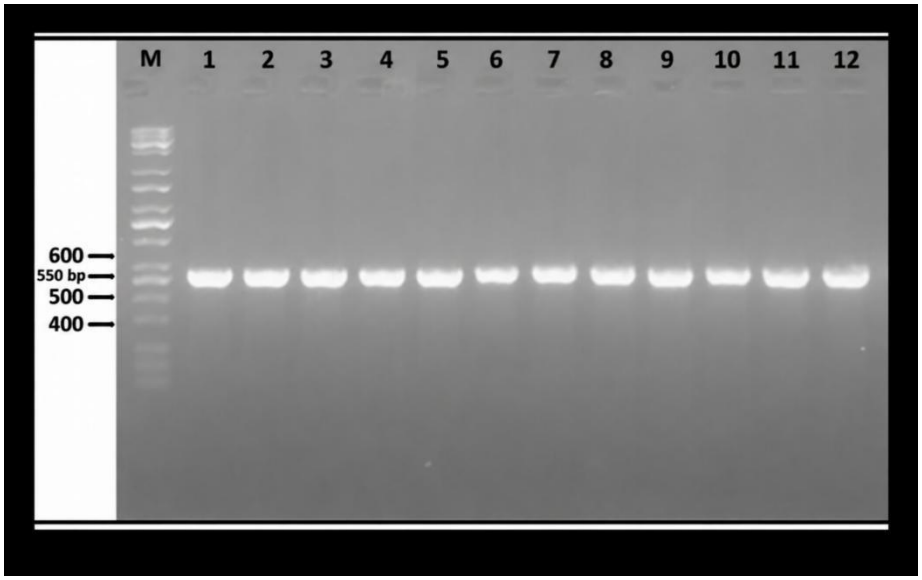



Figure 2. The amplified ITS regions of the rDNA of *A. flavus* isolates along with 1 kb ladder (M) elecrophosed on 1.5% agarose gel.

**Evaluation of the *A. flavus* in vitro pathogenicity on water agar**

The twelve isolates of *A. flavus* exhibited a variation in the infection rate of corn seeds ranging from 17.50% to 85.00% with an average of 44.18% in the result of the laboratory pathogenicity test. Isolate No. (12) had the highest infection rate (85.00%) followed by Isolate No. (1) (60.00%), Isolates No. (6) and (7) (57.50%) each, and Isolate No. (4) (55.10%). Isolates 3, 9, and 5 showed infection rates of 42.50%, 33.00%, and 31.50%, respectively, while isolates 8, 2, and 11 recorded similar rates of 30.50%, 30.00%, and 30.00%, respectively. Among the isolates, isolate 10 had the lowest infection rate of 17.50%, indicating a lesser pathogenicity than the other isolates. However, no infection was recorded in the control group with an infection rate of 0.00%.

Table (4): *In vitro* pathogenicity assessment of *A. flavus* isolates on maize seeds

| Ref. | Isolate | Infection (%) |
|---|---|---|
| **1** | *A. flavus* | 60.00 |
| **2** | *A. flavus* | 30.00 |
| **3** | *A. flavus* | 42.50 |
| **4** | *A. flavus* | 55.10 |
| **5** | *A. flavus* | 31.5 |
| **6** | *A. flavus* | 57.50 |
| **7** | *A. flavus* | 57.50 |
| **8** | *A. flavus* | 30.50 |
| **9** | *A. flavus* | 33.00 |
| **10** | *A. flavus* | 17.50 |
| **11** | *A. flavus* | 30.00 |
| **12** | *A. flavus* | 85.00 |
| | **Control** | 0.00 |
| | **Mean** | 44.18 |

* Each number represents an average of three replicates.

Results from the present study showed that there was a wide array of fungi associated with maize seed, some genera and species were isolated, such as *Aspergillus* spp., *A. niger*, *A. flavus*, *Fusarium* spp., and *Penicillium* spp. *A. flavus* is the most common fungus found which is significant as it is able to colonize maize seeds. This may be attributed to the fact that it is very fast-growing on carbohydrate-rich nutrient sources, and can withstand the harvesting, handling, and storage of grains, especially when humidity and temperature conditions are favorable. Grains can also become infected in the field or during post harvest processing stages from fungal spores in the soil, air and contaminated plant debris. This is similar to the results of Al-Masoodi et al.[14] study in Karbala Governorate finding several fungal genera from stored corn kernels. Aspergillus was the most prevalent followed by Penicillium and Fusarium. This study also showed that several species of Fusarium are present, indicating that corn seeds are an appropriate matrix for the colonization of a variety of fungi. Likewise, Hassan et al. [15] reported several fungi species, such as A. flavus, Fusarium spp., Penicillium spp. and other species of Aspergillus, in stored corn in Iraq. The results agreed with the present study on the diversity of the fungi present on corn seeds and the percentage of each species depends on the source of the samples, storage conditions and the isolation and identification techniques. In 2026, a study conducted in Iraq by Alrawi and Hussein[16] indicated that stored maize had the highest percentage of Aspergillus followed by Penicillium, Fusarium and Alternaria. The study also demonstrated that some *A. flavus* isolates are capable of producing aflatoxin B1, which also indicates the health and economic significance of this fungus, especially when the grains were contaminated for human consumption or used as animal feeds.

The dominance of Aspergillus species in stored maize grains and feed is attributed to this genus's ability to adapt to environmental conditions. It grows in a wide temperature range (12-48°C) and with relatively low moisture content in the field and storage[17]. Aspergillus spp. species produce large numbers of airborne spores, making them among the most common fungal groups infecting maize during harvest and storage [18]. *A. flavus* is one of the species of the genus Most of the types that attack yellow corn grains cause a decrease in nutritional value, germination rates of those grains, and a change in the color and taste of these grains[19].

A study by Sivakaame et al., [20] in India, it was observed that Aspergillus fungi was the predominant group of fungi isolated from maize seeds followed by Fusarium fungi, and multiple species of Aspergillus fungi including *A. flavus* and *A. niger* were present. The findings of the current study are similar to the general trend observed by others who reported that the incidence of Aspergillus fungi on maize seeds varied according to the environment and geographical location and the source of grain. The other way around, the occurrence of the Fusarium and Penicillium fungi in the present isolates is significant for the agriculture and nutrition. Some Fusarium species are plant pathogens and producers of fumonisin while some Penicillium species are involved with grain spoilage in storage and production of mycotoxins. Mohammed et al. [21] reported Fusarium as one of the most commonly occurring genera in a study of local maize grains in Iraq and fumonisin B1 was found in the grains studied. It implies that in the future, assessing the fungal contamination of maize should not be restricted to the identification of fungal species, but also changes in the capacity to produce mycotoxins must be considered.

The results of the lab pathogenicity test revealed that some *A. flavus* isolates were more efficient in inducing infection to maize seeds than others which means differences in seed colonization and pathogenicity under experimental conditions. Isolate No. 12 had the highest rate of infection, and isolate No. 10 had the lowest rate of infection. This could be due to genetic and physiological differences among the isolates, and to the fact that they may be able to produce enzymes that break down cellular components, and secrete compounds that aid in seed tissue penetration and colonization. The variation could also be caused by the different growth rates of the isolates or their ability to produce spores and extracellular enzymes (cellulase, pectinase, protease) that could help to break down structural components of the plants and spread the fungus. In addition, there may be differences in the tolerance of the isolates to the environment which can cause differences in infection levels, even when tested under similar laboratory conditions. The importance of this variation is similar to what was found by Al-Masoodi et al. [14] in Karbala in which they detected several Fusarium species on maize grains, with different diagnostic features. The present study also highlighted the significance of morphological and molecular study of fungal species of the maize. The

data presented in this study do not show the pathogenicity of each isolate of *A. flavus,* but the diversity of corn-related fungi indicates the need to study each pathogen individually.

In the study Mazzoleni et al., [22] it was found that *A. flavus* colonization of fungal seed can affect corn seedling growth and development; this implies that fungal seed colonization may influence seedling growth and development. It is important to study the association of presence of fungi on seeds and the subsequent effect on plant growth. The lack of infection in the control treatment helps confirm the link between infection and fungal isolates, and lessens the chance of the results being attributed to accidental contamination or environmental factors

## CONCLUSION

The study showed that a wide variety of fungal species is associated with maize seeds, of which Aspergillus flavus was the most common fungus. The selected *A. flavus* isolates were successfully amplified into IRS, which was confirmed by molecular analysis. The pathogenicity assay showed significant differences in pathogenicity of the fungal isolates, suggesting their differences in pathogenicity potential. The control treatment showing that no infection was present further substantiates the importance of fungal inoculation in causing seed infection. In overall, the results indicate that the seed-borne fungi may be a threat to the health and quality of maize seeds, especially A. flavus, hence, there is a need to conduct further research on the pathogenic mechanisms of these fungi and how they can be effectively controlled in maize seed.

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