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Identification of atoxigenic *Aspergillus flavus* from stored grains in controlling aflatoxin contamination in maize

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Abstract

Aflatoxins are carcinogenic fungal metabolites produced by several *Aspergillus* species, and current mitigation approaches offer no long-term solution. This study isolated and identified atoxigenic *A. flavus* from fifteen maize-growing areas in Bangladesh and evaluated their efficacy in limiting aflatoxin contamination *in vitro* and *in planta*. A total of 44 *A. flavus* isolates were identified by morphological characteristics and internal transcribed spacer (ITS) sequencing, and atoxigenicity was confirmed by amplification of *nor1, aflR*, and *omtA* genes of the aflatoxin biosynthesis gene cluster. *In vitro*, 38 isolates significantly ($p<0.01$) reduced aflatoxin accumulation by 7% to 92.35% in inoculated maize grains, while 6 isolates increased accumulation by 2.73% to 49.16%. Isolates AF72, AF73, and AF76 increased aflatoxin accumulation despite being confirmed atoxigenic, whereas AF153, AF11, AF13, AF191, and AF90 reduced accumulation despite being confirmed toxigenic. In planta, all isolates significantly ($p<0.01$) reduced aflatoxin accumulation by 80% to 90% except AF01 and AF165; notably, AF72 and AF73, which increased aflatoxin in vitro, reduced it in planta. Further research using cluster amplification pattern (CAP) analysis and single sequence repeat (SSR) genotyping is needed to confirm partial or complete deletion of genes in the aflatoxin biosynthesis gene cluster before these atoxigenic *Aspergillus* isolates can be declared suitable for biocontrol product development.

Keywords: Aflatoxin, Atoxigenic *A. flavus*, Biocontrol, Maize, Bangladesh

Introduction

Maize (Bhutta) or corn, *Zea mays* L, is one of the world's most important cereals, ranking third after wheat and rice in the Poaceae family. In Bangladesh, maize is the third most significant grain crop. In 2010, it covered 2.02 lakh hectares and produced 13.17 lakh metric tons (BBS 2011). Bangladesh was placed 11th in 2019-2020, with an average maize output of 8 tons per hectare. BBS (2019, 2020). The overall planted area was 4.7 lakh hectares, and the total maize production was 40 lakh tons. BBS (2019, 2020). Maize is utilized as feed, food, and fodder in Bangladesh. Maize is utilized in 55 percent of chicken feeds, 40 percent of fish feeds, and just 5% of cow feeds. BBS (2019, 2020). Because of its varied uses, maize has quietly expanded to satisfy future food, feed and other demands, especially given the country's burgeoning cattle and poultry industries. But the most burning issue is that maize is severely affected by mycotoxin, especially aflatoxin.

Aflatoxins are secondary carcinogenic metabolites produced naturally by different species in *Aspergillus* section *Flavi* (Cotty et al. 1994; Frisvad et al. 2018; Norlia et al. 2019) and produce different types of it such as aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1), aflatoxin M_2 (AFM_2) which considered as major aflatoxins (Moretti et al. 2019). Aflatoxins can contaminate wide range of crops such as maize, cassava, sorghum, peanut, cottonseed, chillies, wheat, rice and nuts (Singh and Cotty 2019; Picot et al. 2017; Probst et al. 2007; Kachapulula et al. 2017). IARC lists aflatoxin B_1 as a 1a human carcinogen (IARC 2012). Twenty-eight *Aspergillus* species generate aflatoxins. *Aspergillus* subgenus *Circumdati* section *Flavi* contains aflatoxins-reducing species. *A. flavus* produces AFB_1 and AFB_2, while A. *parasiticus* and A. nomius produce AFB_1, AFB_2, AFG_1 and AFG_2 (Varga et al. 2011), all of them has been identified aflatoxin producing species in foods (Singh et al. 2018; Singh et al. 2020; Yunes et al. 2020). The World Food Program (WFP) established regulatory limits of 10 ng/g for aflatoxins and 1 µg/g for fumonisin (Van Egmond et al. 2007).

Aflatoxins in foods and feeds represent a direct hazard to livestock health and, by lowering livestock production and by transferring it from feed to animal meals like milk, meat and eggs which indirectly harm human nutrition and health, even if milk is the only animal food with substantial aflatoxin carry-over (Frazzoli et al. 2017). The yearly incidence of liver cancer in Bangladesh is estimated by Turna and Wu (2019) at 1311. 62% of 3-year-olds in Dhaka, Bangladesh, had aflatoxin in their blood plasma, suggesting persistent exposure (Mahfuz et al. 2019). Liu and Wu (2010) found that 25–155,000 cases of hepatocellular cancer are linked to aflatoxin exposure. Bangladeshi markets

have significant aflatoxins in maize (Bhuiyan et al. 2013). Globally, South Asia has the greatest prevalence of aflatoxin contamination in maize surpassing EU guidelines, including Bangladesh (Jannat et al. 2022; Gruber-Dorninger et al. 2019). Several cultural, physical, biological and chemical practices have been practising to limit the aflatoxin production in several crops. Pre- harvest and post-harvest technologies such as chemical detoxification, improved sanitation, roasting, washing, UV irradiation, Gamma radiation, Carbon filtration, Thermal procedures, polishing, RNAi silencing, nanoparticles, using genetically resistant plant and sorting have been identified to detoxify the aflatoxins concentration (Matumba et al. 2015; Peng et al. 2018; Mahuku et al. 2019; Mtega et al. 2020; Sani et al. 2014). However, none of those technologies practised individually provides complete protection due to the complexity and the multitude of factors affecting the aflatoxin contamination. Thus, using atoxigenic *A. flavus* from native isolates has been signified the suppression of aflatoxin concentration in maize (Moral et al. 2020).

Biocontrol products made with atoxigenic *A. flavus* have been on the market for more than two decades (Adhikari et al. 2016; Atehnkeng et al. 2008; Cole and Cotty 1990; Dorner and Lamb 2006). The main reason of these atoxigenic *A. flavus* biocontrol products reduce the amount of aflatoxin in crops by eliminating other molds (Cotty and Bayman 1993; Mauro et al. 2018; Senghor et al. 2020). Competitive exclusion is a major and dominating mechanism induced by atoxigenic *A. flavus* isolates as a biocontrol strategy to limit aflatoxin contamination. Competitive interactions can minimize aflatoxin contamination even in high-aflatoxin *A. flavus* groups; besides, complexity of fungal community may influence aflatoxin contamination in plant tissues (Cotty and Bayman 1993). Different *A. flavus* lineages reside in various locations suggesting they may have evolved to niches and competitive advantages in distinct soils, hosts, geographical location and seasons (Bock et al. 2004; Jaime-Garcia et al. 2006; Orum et al. 1997). Atoxigenic *A. flavus* may be able to degrade aflatoxins pre- and postharvest. *A. flavus* and A. *parasiticus* strains that synthesize aflatoxin may degrade it down; however, the particular adaptive functions are not fully understood, it was also thought that genes in the aflatoxin biosynthesis cluster might be involved in the process of degradation (Doyle and Marth 1978a; Hamid and Smith 1987; Huynh et al. 1984).

Aflatoxin production by *A. flavus* is localized on chromosome III, 80 kb from the telomere. Aflatoxin production requires regulatory proteins and enzymes. Biosynthesis involves 30 genes, 27 enzymatic steps, and 15 structural intermediates. Early pathway (*aflA, aflB, aflC, aflD, aflR, aflJ)* genes, intermediate pathway (*aflE, aflG, aflH, aflI, aflK, aflL, aflM, aflN, aflV, aflW*) genes, and late pathway (*aflO, aflP, aflQ*) genes (Rao et al. 2020). For the detection

of aflatoxins and aflatoxigenic molds, numerous traditional and chromatography-based techniques, such as thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC), etc., are available (Bacaloni et al. 2008). To test for aflatoxigenic fungi, Polymerase Chain Reaction (PCR) offers an advantage over other techniques. Several genes involved in the biosynthetic route of aflatoxin production, including *afl R, nor-1, omt-1,* and *ver-1*, have been targeted in this direction (Criseo et al. 2001). Different mutations in *A. flavus* isolates have significant influences on aflatoxin production. These mutations range from point mutation to partial or full deletions of the aflatoxin biosynthesis gene cluster (Donner et al. 2010; Ehrlich et al. 2007; Adhikari et al. 2016; Chang et al. 2005). One or more gene deletions or mutations result in a non-aflatoxigenic phenotype. Overall, only a small number of studies have looked at how well atoxigenic *A. flavus* can break down aflatoxins (Cotty and Bayman 1993; Doyle and Marth 1978b; Rao et al. 2020; Xing et al. 2017). The biocontrol mechanism may entail the breakdown of aflatoxin, suppression of aflatoxin formation, competition for nutrition, and the regulation (Cotty and Bayman 1993; Huang et al. 2011; Rao et al. 2017). Agbetiameh et al. (2020) examined two aflatoxin biocontrol products termed Aflasafe GH01 and Aflasafe GH02 that were elicited from Ghanaian soil able to combat aflatoxin. Serenade Max® and Vacciplant® significantly reduced *A. flavus* sporulation *in vitro* (48.2–63.1%) (Lagogianni and Tsitsigiannis 2019). In the US, the aflatoxin biocontrol product AF36 has been successful in several crops (Ortega-Beltran et al. 2018). Mauro et al. (2018) employed an AFIT-01 biocontrol product consisting of locally isolated atoxigenic *A. flavus* to reduce mycotoxin contamination in maize by 83.7% to 94.87%. Biocontrol formulations containing atoxigenic fungi native to target locations should be more successful due to their adaptability to the environment, cropping system, climate, and soil (Atehnkeng et al. 2016; Mehl et al. 2012). As no prior research has been practised to evaluate the atoxigenic *A. flavus* as a biocontrol agent to minimize aflatoxin contamination as well as no such regulatory limit has been imposed by Bangladesh despite the establishment of Bangladesh Food Safety Authority (BFSA), thus the present study focused to accomplish the following objectives: To isolate *A. flavus* isolates from maize grain and identify the atoxigenic *A. flavus* by PCR using aflatoxin biosynthesis gene markers and To evaluate the efficacy of atoxigenic *A. flavus* as a biocontrol agent limiting aflatoxin contamination.

Materials and Methods

Isolation and identification of the *A. flavus* from maize grains

Aflatoxin-producing *A. flavus* isolates were isolated from ground maize which recovers fungi colonizing and infecting internal tissues. A representative sample (20g) was ground with a blender so that 75% can pass through a 20-mesh screen, then the sub sample portion was mixed thoroughly. 5 g of ground maize was added in to a 250 ml Pyrex bottle containing autoclaved water with 0.001% TWEEN-80. Once all samples were weighed out, bottles were placed on a shaker for 20 min at 175 rpm. After mixing, sample bottles were put in a biosafety cabinet and 100 µl aliquots were spread onto an isolation medium (3 plates per sample) using a cell spreader to evenly distribute the suspension across the surface of the Clean Up (CU) agar plate (Callicott et al. 2018) **(Supplementary Figure S2)**. Once all of the isolation plates were inoculated, the plates were incubated for 3 days at 31˚C in the dark. At the end of the incubation period, colonies were counted, and *Aspergillus* isolates were picked from plates. The candidate colonies were then transferred to a Potato Dextrose Agar (PDA) growth medium for purification and incubated at 31°C for 7-10 days (Callicott et al. 2018) (**Supplementary Figure S1**). Then *A. flavus* isolates were preserved in cryovial with sterile water in which three representative blocks (3mm) were placed collecting with needle (Garber et al. 2012). Colonies of *A. flavus* isolates were identified based on morphological characters and also identification was performed under compound microscope by using a temporary slide.

Molecular based identification of *A. flavus*

PDA agar plates were prepared with 2% NaCl by pipetting 10µl spore suspension to a central wall in the agar. Then the plates were incubated at 31ºC for 5-7days. The cultures were then harvested with a spatula and transferred to Eppendrof tubes containing TWEEN-80. The tubes were then centrifuged for 5 minutes at 8000 × g. Then, 450µl lysis buffer was added to each tube and vortexed to suspend spores. Tubes were placed in water bath at 60ºC and vortexed for 10 seconds after 30 minutes and returned to incubation for another 30 minutes, then vortexed again after the second 30minutes. The tubes were then centrifuged again for 30 minutes at 14000 × g. 370µl supernatant was transferred to a new labeled sterile tube without disturbing the pellet. 370µl 4M ammonium acetate (NH4OAc) was added from the refrigerator and the tubes were mixed upside down for several times. 100% 370µl ice-cold ethanol was added and mixed tubes by inverting. Later, the tubes were kept at -20ºC for 30minutes at the

refrigerator. Then, the tubes were spined at maximum speed for 5 minutes. The supernatant was poured off and kept the tubes open on a paper with upside down position. Finally, 25µl sterile water was added to the dry labelled tubes and vortexed to resuspended DNA (Callicott et al.2018).

Detection of *A. flavus* by sequencing Internal Transcribed Spacer (ITS) region

The isolated DNA samples were amplified using the forward primer ITS1-5.8S (5'-GGAAGTAAAAGTCGTAACAAGG-3') and the reverse primer rDNA-ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990). Initial denaturation at 95°C for 5 minutes was followed by 34 cycles at 95°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute, and final elongation at 72°C for 6 minutes. PCR reactions were carried out in a 25µl reaction volume that contained 12.5µl master mix (Promega, GoTaq® Green Mastermix) 1µl ITS1, 1µl ITS4, 9.5µl Nuclease-free water, and 1µl of templet DNA (100ng/l). PCR results were stained with ethidium bromide and photographed in a 2% agarose gel using a Gel documentation system. PCR results were then sequenced using ITS1 primers with the assistance of a Biotech company in Macrogen, Korea. Finally, Chromas Software imported Sequence data. Blast (Basic Local Alignment Search Tools) and Genebank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to analyse sequence data. BLAST GenBank was used to acquire reference sequences with high homology.

Detection of atoxigenic *A. flavus* by nor-1, *aflR* and *omtA*

The primers *nor1* FP (5'-ACCGCTACGCCGGCACTC TCGGCAC-3') and *nor1* RP (5'-GTTGGCCGCCAG CTTCGACACTCCG-3') were used to amplify a 400 bp fragment of norsolorinic acid reductase (Criseo et al. 2001); *omtA* FP (5'-GGCCCGGTTCTGGCTCCTAAGC3') and all of these *A. parasiticus* genes' nucleotide sequences are available at NCBI GenBank under the accession numbers L27801 (*nor1*), SRRC 2043 (*aflR*), and SRRC 143 (*omt-1*) (Ibrahim et al. 2016). The reaction volume for the PCR was 15µl, and the components were 7.5µl master mix (Promega, GoTaq® Green Mastermix), 1µl forward primer, 1µl reverse primer, 4.5µl nuclease-free water, and 1µl extracted DNA as template (with a total concentration of 100 ng of genomic DNA per reaction). *Nor1* was subjected to the following PCR conditions: initial denaturation for 5 min at 94°C, 35 cycles at 94°C for 30 s, 67°C for 30s, and 72°C for 30 s, and finally final elongation for 10 min at 72°C (Criseo et al. 2001). For *omtA* and *aflR*, the PCR conditions were initial denaturation for 10 minutes at 95°C, followed by 30 cycles at 94°C for 1 minute, at 65°C for 2 minutes, and at 72°C for 2 minutes, and finally, final elongation for 5 minutes at 72°C (Ibrahim et al. 2016). In a

gel documentation system, PCR products were separated by electrophoresis on a 1% agarose gel with 0.5% ethidium bromide in 1X TBE buffer (G: box, Syngene, India). The molecular size marker for the examination of the agarose gel will be 1 kb plus DNA Ladder.

Assessment of atoxigenic *A. flavus* in *in vitro* maize grains

At first, *A. flavus* was grown on PDA for 7days then the conidia were harvested with the help of spatula washed with ethanol and conidial suspension was prepared in sterile water. The conidial suspension was then filtered through sterile Cheech cloth. Then the number of conidia (10^6 spores/ml) were counted with the help of Marienfield Haemocytometer (Cotty 1994). The number of spores was then counted using the formula below:

$$\text{Cells per } 1\ \mu\text{l solution} = \frac{\text{Number of cells}}{\text{Counted area } (\text{mm}^2) \times \text{Chamber depth (mm)} \times \text{Dilution}} \quad ..(1)$$

Healthy, undamaged maize kernels were separated. Moisture content of maize kernels was measured. 250gmaize was added to 1liter Niagene roller bottle. Water content of grains was adjusted to 20% using the following formula. However, 1% moisture is the same as 1ml per 100gm maize.

$$\text{Volume added water} = \frac{\text{Mass grain} \times (\%\text{Moisture desired} - \%\text{Moisture measured})}{\text{Water desired} \times \text{Water measured}} \quad ..(2)$$

Then, 10 g maize kernels were added to 250 ml flasks. The flask was covered with aluminium foil as well as a control flask was also prepared. The covered flasks were autoclaved at 121°C for 20minutes. After return to room temperature moisture content was measured from all flask with control flask. Then, the maize was inoculated with fungal suspension (containing $1x10^6$ conidia/ml of *A. flavus*) with appropriate volume to bring total moisture to 25% using the equation above. Water was used as a negative control. Before transferring into incubator flasks were tightly capped. Inoculate maize was incubated at 31ºC in the dark for 7 days in the incubator. Then, aflatoxin concentration was measured.

Assessment of atoxigenic *A. flavus* in in planta with an artificial inoculation

At first, the soil was sterilized with 4% formalin solution @ 500 ml/ cft soil. The soil was then covered with black polyethene sheet and left for 48 hours. Then, the soil was spread out on a paper sheet and left for two weeks. 50 pots were used to sow the maize seeds. Pots were manually sown with maize seeds (three seeds per pot). After sowing

proper irrigation, fertilization was done. After three to four weeks of planting, only one seed was left in the pot for cob formation. Inoculation of *A. flavus* fungal suspension was prepared by following the method section. Each growing ear was once damaged in the center during the mid-silking stage by inserting a cork borer (3 mm in diameter) through the cob husk to a depth of 5 mm. With a pipetter, 10 µl of spore suspension containing $1x10^6$ conidia/ml *A. flavus* isolates were injected into each ear (Abbas et al. 2006). (**Supplementary Figure S3**).

Sample collection, preparation and determination of total aflatoxins in maize grains

At maturity, each plot's inoculated cobs were picked and allowed to dry in the sun for 14 days. The grains that were immediately around and abutting the point of inoculation made up the first group of kernels (inner ring). The kernels immediately surrounding the inner ring made up the second group of grains (outer ring). With the aid of forceps, the inner and outer ring kernels were separated, placed in separate envelopes, and kept at 4°C until analysis. A representative sample was obtained and grounded by blender so that 75% of it could pass through a 20-mesh sieve. The sub-sample fraction was then completely blended. 50 g of ground samples were weighed into a spotless, airtight conical flask. The powdered sample was mixed with 250 mL of methanol extraction solution (70% methanol diluted in water). The flask was then sealed. After that, the sample-containing conical flask was shaken for three minutes. After giving the sample time to settle, the top layer of the extract was filtered using Whatman #1 filter paper, and the filtrate sample was then collected. At 1:20, distilled water was used to dilute the produced extract. Without additional preparation, the sample was prepared for testing (**Supplementary Figure S4**). Then, an enzyme-linked immunosorbent assay (ELISA) was performed by the AgraQuant aflatoxins test kit (Romer Lab, USA). In dilution wells, 200 µl of the conjugate solution was pipetted out. The dilution wells received 100µl of each extract from the standard and sample. After thoroughly mixing the mixture, 100 µl of it (conjugate, standard, or samples) was poured into antibody-coated wells. Next, the plate was incubated for 15 minutes while gently shaken. The plate was rinsed five times with distilled water. The plate was then dried using a tap. A substrate solution containing 100µL was pipetted into wells covered with antibodies. For five minutes, the plate was shaken during incubation. Pipetting 100µl of stop solution into antibody-coated wells. Each well's absorbance was measured at 450 nm and 630 nm for the differential filter **(Supplementary Figure S5)**.

Data Analysis

The data on various parameters were statistically analysed using ANOVA (analysis of variance) to find out the variation resulting from experimental treatments, the means and standard deviation were also calculated by using MINITAB software (version 19) (Ramesh 2009).

Results

Isolation and morphological identification of *A. flavus* from the stored maize grain samples

A. flavus isolates were isolated from the CU agar media (**Figure 1A**) where *A. flavus* colonies were grown and then a representative colony was picked up for subculture and purified in PDA media (**Figure 1B**). *A. flavus* isolates were morphologically identified by observing olive-green colony colour on culture plate and their microscopic structures under compound microscope which showed radiating conidial heads while the conidiophores appeared rough (**Figure 1C**). However, following above mentioned techniques overall forty-four isolates of *A. flavus* were identified and four representative morphological pictures of *A. flavus* were showed in **Supplementary Figure S1**.

Identification and confirmation of *A. flavus* using ITS primers

One representative isolate, AF02, was identified PCR amplification of ITS region using ITS1 and ITS4 primers because globally similar type of morphological characteristics was identified by ITS1 and ITS4 and the isolate *A. flavus* showed an amplicon size of 600bp which confirmed the fungal isolate. Sequence result of that PCR product revealed that AF02 obtained from Panchagarh showed 99.33% homology with *A. flavus* strain SU-16 (accession number MT680400.1) (**Figure 1D**, **Table 1**). These results might be confirmed that all of the morphologically identified isolates were *A. flavus*.

Confirmation of atoxigenic *A. flavus* using *nor1*, *aflR* and *omtA* genes

To identify atoxigenic *A. flavus*, one gene was selected as regulatory gene (*aflR*) and other two genes (*nor1*, *omtA*) were selected as structural genes from aflatoxin biosynthesis gene cluster. Atoxigenic *A. flavus* was confirmed by *nor1*, *aflR*, *omtA* primers by observing amplification pattern in the region of 400bp, 1032bp and 1024bp, respectively in all isolates (**Figure 2**). Seven isolates amplified with a band size of 400bp using *nor1* primer (**Figure 2A**). No amplification was observed among forty-four isolates using *aflR* primer (**Figure 2B**), and six isolates were

amplified with a band size of 1024bp using *omtA* primer (**Figure 2C**). To compare the atoxigenic amplification, six isolates were amplified which were presumed to be toxigenic isolates as some of those isolates showing specific band (**Figure 2ABC**).

Screening of atoxigenic *A. flavus* isolates based on PCR banding patterns

Total forty-four isolates were screened out to separate atoxigenic isolates by PCR with *nor1*, *omtA*, *aflR* primers. In case of confirmation of atoxigenic isolates, only those showed no amplification with all of these three primers have been counted as atoxigenic isolates while confirmation of toxigenic isolates was determined by observing any amplification by these primers (*nor1*, *omtA*, *aflR*). Among all isolates, thirty-three isolates showed no amplification with *nor1*, *omtA*, *aflR*. Conversely, eleven isolates showed positive amplification with any of these primers (either *nor1*, or *aflR* or *omtA*). Isolates AF11, AF13, AF165, AF168 and AF01were only amplified with *nor1* primer, however, these isolates showed no amplification with *aflR* and *omtA* primers, thus these isolates considered as toxigenic isolates. Four isolates, AF153, AF90, AF31_2 revealed positive amplification only with the *omtA* primer. Moreover, isolate AF191 and AF101_2 showed positive amplification with *nor1* and *omtA* primers (**Table 2**).

Assessment of *A. flavus* in inoculated maize in *in vitro*

The minimal amount of aflatoxin concentration was detected in maize grains inoculated with isolate 2.97 µg/kg (AF71) followed by 3.10 µg/kg (AF75), 3.34 µg/kg (AF44) and 3.42 µg/kg (AF191) which were able to significantly ($p < 0.01$) reduce 92.35%, 92.03%, 91.40% and 91.19%, respectively compared to uninoculated maize (**Table 3**). Lower moderate level of aflatoxin concentration was detected in maize grains inoculated with isolate AF202 followed by AF03, AF74, AF11, AF31_2, AF90, AF02, AF153, AF6, AF173 and AF101_3 which correspond to the reduction of 85.43%, 78.29%, 77.70%, 77.60%, 77.35%, 76.94%, 76.78%, 76.35%, 76.24%, 75.96% and 74.99% aflatoxin accumulation compared to uninoculated maize. Some maize grains inoculated with isolates (AF01, AF76, AF168, AF73, AF101_2, AF72) significantly increased total aflatoxin concentration. Besides, some isolates which were previously considered as atoxigenic isolates (phenotypically) (**Table 2**), they are still increasing aflatoxin concentration (genetically) such as AF72, AF73 and AF76 increased 2.73%, 12.98% and 26.12%, aflatoxin, respectively. Conversely, some isolates (AF153, AF11, AF13, AF191, AF90 and AF165) which were considered before as toxigenic isolates (**Table 2**), were able to reduce 76.33%, 77.60%, 68.77%, 91.79%, 76.94%

and 2.73%, aflatoxin, respectively. However, a few isolates considered as toxigenic isolates ultimately increased the total aflatoxin concentration for instance, maize grains inoculated with isolates AF01, AF168, AF185, AF101_2 increased the total aflatoxin concentration up to 49.16%, 14.26%, 17.51% and 3.17%, respectively.

Reduction of total aflatoxin using atoxigenic *A. flavus* isolates in planta maize cob

Similar to *in vitro* assessment, same isolates were evaluated in *in planta* inoculation to measure the total aflatoxin concentration and the efficacy level of aflatoxin reduction. The results from **Table 4** indicated that the 44 isolates significantly ($p < 0.01$) reduced the total aflatoxin concentration compared to uninoculated maize.

Almost all isolates showed better performance than *in vitro* assessment of aflatoxin reduction. More than 90% reduction of AFs was observed by nineteen isolates, among them isolates AF72 and AF73 were previously identified as increasing aflatoxin concentration in *in vitro* but they are still highly capable of reducing aflatoxin concentration in field. Moreover, 20 isolates were significantly reduced more than 80% reduction of total aflatoxin. Apart from the highest reduction of total aflatoxin concentration, cob inoculated with the isolates such as AF01 and AF165 showed less reduction than all other isolates and that was 67.10% and 74.65%, respectively. Adding to this, grains inoculated with AF01 was previously showed increasing total aflatoxin in *in vitro* assessment (**Table 3**).

Discussion

The experiments were conducted at Plant Bacteriology and Biotechnology Laboratory, Department of Plant pathology, Bangladesh Agricultural University to isolate and identify atoxigenic *A. flavus* from stored maize grain as well as to evaluate their efficacy in reducing aflatoxin contamination in inoculated maize.

Morphological identification of *A. flavus* was confirmed by observing yellowish to green colour on PDA comparing with *A. niger* which showed dark woody colour and their microscopic views were biseriate head with globose vesicle, these findings were in accordance with the study conducted by Afzal et al. (2013). Another study confirmed that on PDA media, in the beginning, mycelial colour of *A. flavus* was white but after three days of incubation the colony colour was transformed into olive-green (Khan et al. 2020). Previously, White et al. (1990) confirmed the fungal isolate with primers ITS1 and ITS4 with an amplicon size of 600bp in accordance with this study.

For the identification of toxigenic and non-toxigenic strains of *A. flavus* and *A. parasiticus*, molecular approaches are commonly used because to their increased sensitivity and specificity, which allows for a quick diagnosis (Ritter

et al. 2011; Wei et al. 2014; Yin et al. 2009; Reichard et al. 2000; Jiang et al. 2009). Polymerase Chain Reaction was used to detect toxin producing *A. flavus* by *nor1*, *aflR*, *omtA* gene-based marker which were able to synthesize enzymes involved in the aflatoxin biosynthesis pathway (Ibrahim et al. 2016). Regulatory Factors and vital enzymes were encoded by these genes in AFs biosynthesis pathway. In this study, to identify the toxigenic and atoxigenic isolates associated with maize grains, PCR was performed by primers *nor1*, *aflR*, *omtA* and isolates were confirmed as primarily toxigenic and atoxigenic based on their amplification pattern. Likewise, earlier studies confirmed that these genes *nor1*, *apa-2*, *omtA* were responsible for positive amplification of *Aspergillus* species such as *A. parasiticus* and *A. flavus* (Shapira et al. 1996). Another similar finding was detected by Criseo et al. (2001) using *aflR*, *omt-A, ver-1* and *nor1* genes showing clear detection of aflatoxigenic strains of *A. parasiticus* and *A. flavus* via multiplex PCR. However, isolates of *A. niger* were not found positive by these genes as they were not producing aflatoxins rather produce Ochratoxin A (Schuster et al. 2002).

In this study, majority of the isolates confirmed as atoxigenic as no amplification pattern was observed by these genes while some isolates showed only positive amplification with one of these genes, both regulatory and functional genes. As according to Chang et al. (2005), atoxigenic *A. flavus* isolates are primarily linked with deletions of a portion or deletion of the full aflatoxin gene cluster which is similar with this experiment. Total thirty-three isolates were confirmed as atoxigenic isolates as they showed complete deletion of *nor1*, *aflR*, *omtA* genes in the aflatoxin biosynthesis cluster. The atoxigenic characteristic of *A. flavus* AF36 isolate cotton seed is due to mutations in the aflatoxin gene pksA (Criseo et al. 2001). Therefore, deletion analysis within the aflatoxin gene cluster may be an efficient tool for the quick identification of atoxigenic *Aspergillus* strains (Houshyarfard et al. 2014). Eleven toxigenic isolates were identified by the amplification of one of these genes (*nor1*, *aflR*, *omtA*) as these genes are responsible for triggering several functions such as Yu et al. (2012) described that *Ver-1* codes for versicolorin A dehydrogenase, which transforms versicolin A to sterigmatocystin. The *omtA* gene is involved in the conversion of sterigmatocystin to O-methyl sterigmatosystin, whereas the *nor1* gene codes for a reductase that may convert norsolorinic acid to averantin. In *A. flavus* and *A. parasiticus*, the regulatory genes that activate the pathway genes are *aflR* and *apa- 2*, respectively.

In vitro results indicated that the thirty-five isolates conceded that they were able to diminish total aflatoxin concentration ranging from 2.63% to 85.43%, among these thirty-five isolates, seven isolates (AF153, AF31_2, AF11, AF13, AF191, AF90, AF165) were confirmed previously as toxigenic isolates by nor, *aflR*, *omtA* but still

these isolates were able to reduce total aflatoxin reduction in inoculated maize to a large extent varying from 2.63% to 91.19%. The possible reason behind this that the several kinds of mutation may have rendered the inactivation of aflatoxin biosynthesis pathway of these strains and substitution of some based which ultimately triggered the synthesis of non-functional products (Criseo et al. 2001) revealed that even certain strains expressing *aflR* did not generate aflatoxins although structural gene, *aflR*, plays a significant role which influences the activity of other structural genes, such as *omt-A, ver-1*, and *nor1* in the aflatoxin biosynthesis pathway (Woloshuk et al. 1994; Chang et al. 1999).

On the other side, grains inoculated with isolates AF72 and AF73 increased total aflatoxin concentration 2.73% and 12.98%, respectively even though they were considered as atoxigenic isolates. This may happen due to the degradation of aflatoxin such as Cotty and Bayman (1993), who found out that the non- aflatoxigenic strain AF36 does not breakdown aflatoxin. Large deletions in the aflatoxin gene cluster accounted for additional causes of the atoxigenic character of *Aspergillus* spp. PCR tests have shown that *A. flavus* strains containing the full aflatoxin gene cluster are incapable of producing aflatoxins (Criseo et al. 2001). In contrast, Xing et al. (2017) showed that two non-aflatoxigenic *A. flavus* strains JZ2, GZ15 effectively breakdown AFB_1 and two novel metabolites were produced. Hamid and Smith (1987) showed the enzyme cytochrome P-450 monooxygenases generated by *A. flavus* are involved in the breakdown of aflatoxin. According to Doyle and Marth (1978b) degradation of aflatoxin *A. flavus* is in close association with the strains capacity to generate aflatoxin and is strain dependent.

In planta results showed that the significant reduction of total aflatoxin concentration ranging from 2.63% to 92.35% considering all isolates which was much higher than *in vitro* analysis of aflatoxin reduction. The variation occurs due to high levels of genotypic and phenotypic variety within *A. flavus*, which includes differential synthesis of cyclopiazonic acid, aflatoxins, sclerotia, and pectinase (Cleveland and Cotty 1991; Cotty et al. 1990) as well as ability to use aflatoxin as a nutrition source (Mehl & Cotty 2013). The dynamics of aflatoxin degradation is controlled by favored carbon sources and the rate of aflatoxin degradation, which seem to be the greatest within the first few days following inoculation of maize with atoxigenic *A. flavus* (Maxwell et al. 2021) as well as aflatoxin degradation does not need the presence of genes from the aflatoxin biosynthesis cluster. However, previous research as suggested that enzymes involved in aflatoxin production may also play a role in degradation. As a result, it is unclear which enzymes are involved in aflatoxin degradation by atoxigenic *A. flavus* or what the exact degradation products are (Doyle and Marth 1978b; Hamid & Smith 1987). It has been argued on several contexts that indigenous

endemic atoxigenic genotypes are preferable to introduced genotypes owing to their greater adaptability to the target agroecosystem and related niches (Probst et al. 2011). Two native registered biocontrol product Aflasafe GH01 and Aflasafe GH02 were able to diminish 98.6% and 99.5% aflatoxins in crops showing how reliable biocontrol based on atoxigenic strains is (Agbetiameh et al. 2020). Mauro et al. (2018) reported that in Italy, AFIT-01 biocontrol product made of locally isolated atoxigenic *A. flavus* was applied to prevent the mycotoxin contamination in maize revealing 83.7% to 94.8% abatement in field level. As long as pre-harvest application of atoxigenic *A. flavus* biocontrol product are serving tremendous effect in reducing aflatoxin in USA, Italy, Sub-Saharan Africa, Pakistan. However, further research should be conducted to identify more atoxigenic *A. flavus* through multiplex PCR, SSR genotyping as well as to understand the mechanism involving aflatoxin degradation, synthesis and enzymatic function in biosynthesis genes.

Assessment of atoxigenic *A. flavus* isolates in *in planta* revealed that 19 isolates were able to reduce more than 90% of the total aflatoxin accumulation and 20 isolates were able to diminish more than 80% of the total aflatoxin accumulation. However, in *in vitro*, grains inoculated with isolate AF72 and AF73 were increased total aflatoxin contamination but in *in planta* they were able to reduced total aflatoxin contamination. To conclude that the native atoxigenic *A. flavus* isolates isolated from maize grains were promising in reducing total aflatoxin accumulation in both *in vitro* and *in planta*. Apart from that some behavioral changes were observed among the efficacy level of atoxigenic isolates as toxigenic and toxigenic isolates as atoxigenic in both *in vitro* and *in planta* due to several reasons including mutation of genes, substitution of base pairs, enzymatic processes involved in aflatoxin accumulation and factors which are still not clearly identified. Therefore, more future research focusing SSR and CAP analysis will be required to confirm either partial or complete deletion of genes in aflatoxin biosynthesis gene cluster before being declared as an atoxigenic *Aspergillus* isolates for developing biocontrol product.

Statements and Declarations

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Ethical Approval

The authors confirm that all experiments were performed in accordance with the laws and regulations of Bangladesh. This article does not contain any studies involving human participants or animals.

Data Availability

All data supporting the conclusions of this article are included within the article and its supplementary files. Raw datasets are available from the corresponding author upon reasonable request.

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Conflicts of interest

None.

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List of Figure Captions

Figure 1. Isolation and morphological identification of *A. flavus* isolates. **A)** CU agar plate showed colonies of *A. flavus* isolates. **B)** Velvety yellowish to olive green colonies of *A. flavus* on PDA, **C)** Vesicle with less conidial ornamentation with conidiophores of *A. flavus*. **D)** PCR based identification of representatives *A. flavus* isolate using ITS primers. M, molecular marker; AF02, *A. flavus* isolate; W, water (negative control).

Figure 2. A) PCR amplification of *nor1* gene from the genomic DNA of the *A. flavus* isolates with a band size of 400bp, M: 1 kb plus DNA ladder, AF11, AF13, AF105, AF151, AF101_3, AF14, AF102, AF76, AF44, AF71, AF75, AF173, AF6, AF10, AF67, AF1, AF2, AF3, AF4, AF5, AF202, AF212, AF7, AF72, AF73, AF74, AF79, AF119, AF153, AF27, AF23, AF90, AF184, AF191, AF31_2, AF101_2, AF31_3, AF152, AF34, AF165, AF166, AF168, AF176, AF85. Five isolates AF6, AF32, AF114, AF115 and AF 160 were used as a positive control by amplified by *nor1* gene.

Figure 2. B) PCR amplification of *aflR* gene from the genomic DNA of the *A. flavus* isolates with a band size of 1032bp, M: 1 kb plus DNA ladder, AF11, AF13, AF105, AF151, AF101_3, AF14, AF102, AF76, AF44, AF71, AF75, AF173, AF6, AF10, AF67, AF1, AF2, AF3, AF4, AF5, AF202, AF212, AF7, AF72, AF73, AF74, AF79, AF119, AF153, AF27, AF23, AF90, AF184, AF191, AF31_2, AF101_2, AF31_3, AF152, AF34, AF165, AF166, AF168, AF176, AF85. Six toxigenic isolates AF6, AF32, AF46, AF114, AF115 and AF 160 were used as a positive control amplified by *aflR* gene.

Figure 2. C) PCR amplification of *omtA* gene from the genomic DNA of the *A. flavus* isolates with a band size of 1024bp, M: 1 kb plus DNA ladder, AF11, AF13, AF105, AF151, AF101_3, AF14, AF102, AF76, AF44, AF71, AF75, AF173, AF6, AF10, AF67, AF1, AF2, AF3, AF4, AF5, AF202, AF212, AF7, AF72, AF73, AF74, AF79, AF119, AF153, AF27, AF23, AF90, AF184, AF191, AF31_2, AF101_2, AF31_3, AF152, AF34, AF165, AF166, AF168, AF176, AF85. Six toxigenic isolates AF6, AF46, AF114, AF115 were used as a positive control amplified by *omtA* gene**.**

Tables

Table 1. Representative *A. flavus* isolate identified by homology search of the sequence of ITS region using the BLAST program.

Isolate ID	Location	Closet Relatives	Accession Number	Identity	Homology (%)
AF02	Panchagarh	*Aspergillus flavus* strain SU-16	MT680400.1	591/595	99.33

Table 2. Screening of atoxigenic *A. flavus* isolates by *nor1, aflR, omtA*

Isolate No	Location	*nor1*	*aflR*	*omtA*	Atoxigenic/toxigenic
AF6	Panchagarh	-	-	-	Atoxigenic
AF10	Panchagarh	-	-	-	Atoxigenic
AF01	Panchagarh	+	-	-	Toxigenic
AF02	Panchagarh	-	-	-	Atoxigenic
AF03	Panchagarh	-	-	-	Atoxigenic
AF04	Panchagarh	-	-	-	Atoxigenic
AF07	Panchagarh	-	-	-	Atoxigenic
AF168	Panchagarh	+	-	-	Toxigenic
AF176	Panchagarh	-	-	-	Atoxigenic
AF5	Panchagarh	-	-	-	Atoxigenic
AF151	Thakurgaon	-	-	-	Atoxigenic
AF14	Thakurgaon	-	-	-	Atoxigenic
AF153	Thakurgaon	-	-	+	Toxigenic
AF152	Thakurgaon	-	-	-	Atoxigenic
AF27	Dinajpur	-	-	-	Atoxigenic
AF23	Dinajpur	-	-	-	Atoxigenic
AF31_3	Dinajpur	-	-	-	Atoxigenic
AF34	Dinajpur	-	-	-	Atoxigenic
AF31_2	Nilphamari	-	-	+	Toxigenic
AF11	Rangpur	+	-	-	Toxigenic
AF13	Rangpur	+	-	-	Toxigenic
AF44	Rangpur	-	-	-	Atoxigenic
AF191	Lalmonirhat	+	-	+	Toxigenic
AF67	Gaibandha	-	-	-	Atoxigenic
AF76	Bogura	-	-	-	Atoxigenic
AF71	Bogura	-	-	-	Atoxigenic
AF75	Bogura	-	-	-	Atoxigenic

AF173	Bogura	-	-	-	Atoxigenic
AF72	Bogura	-	-	-	Atoxigenic
AF73	Bogura	-	-	-	Atoxigenic
AF74	Bogura	-	-	-	Atoxigenic
AF79	Bogura	-	-	-	Atoxigenic
AF90	Natore	-	-	+	Toxigenic
AF185	Natore	-	-	+	Toxigenic
AF165	Natore	+	-	-	Toxigenic
AF166	Natore	-	-	-	Atoxigenic
AF105	Jashore	-	-	-	Atoxigenic
AF101_3	Jashore	-	-	-	Atoxigenic
AF102	Jashore	-	-	-	Atoxigenic
AF101_2	Jashore	+	-	+	Toxigenic
AF85	Jashore	-	-	-	Atoxigenic
AF202	Chuadanga	-	-	-	Atoxigenic
AF119	Chuadanga	-	-	-	Atoxigenic
AF212	Cumilla	-	-	-	Atoxigenic

598 ‘’+” indicates positive amplification of any of these genes (*nor1*, *aflR*, *omtA*),

599 ‘’-” indicates no amplification of any of these genes (*nor1*, *aflR*, *omtA*)

600

Table 3. Efficacy of atoxigenic *A. flavus* isolates in reducing total aflatoxin concentration in inoculated maize in *in vitro*

Isolate no	Isolates obtained from the Area	Total aflatoxin (ng/g)	Reduction of %Total Aflatoxin	Comments
Control		38.85	0.00	Nill
AF6	Panchagarh	9.23 ± 2.00m-p	76.24 ± 0.89c-f	Decrease
AF10	Panchagarh	28.19 ± 1.00g	27.44 ± 0.42pq	Decrease
AF176	Panchagarh	10.27 ± 3.00m-o	73.56 ± 0.19e-h	Decrease
AF02	Panchagarh	9.02± 2.00m-p	76.78 ± 0.59cd	Decrease
AF01	Panchagarh	57.95± 1.00a	49.16 ± 0.63n	Increase
AF04	Panchagarh	10.22± 1.00m-p	73.69 ± 0.99e-g	Decrease
AF03	Panchagarh	8.44± 1.57n-p	78.29 ± 1.01c	Decrease
AF07	Panchagarh	10.69± 1.07l-o	72.49 ± 0.99gh	Decrease
AF5	Panchagarh	13.46± 1.57j-m	65.34 ± 1.1k	Decrease
AF168	Panchagarh	44.39± 1.57cd	14.26 ± 1.3s	Increase
AF153	Thakurgaon	9.19± 0.90m-p	76.35 ± 0.77c-e	Decrease
AF14	Thakurgaon	12.66± 1.26k-o	67.42 ± 0.44jk	Decrease
AF151	Thakurgaon	35.99± 2.05ef	7.37 ± 1.46t	Decrease
AF152	Thakurgaon	17.81± 1.23h-j	54.17 ± 0.73m	Decrease
AF31_3	Dinajpur	12.30 ± 1.35k-o	68.33 ± 1.32i-k	Decrease
AF34	Dinajpur	14.85 ± 1.45i-l	61.77 ± 0.67l	Decrease
AF27	Dinajpur	12.34±1.25k-o	68.25 ± 0.49i-k	Decrease
AF23	Dinajpur	10.37± 1.31l-o	73.30 ± 1.23f-h	Decrease
AF31_2	Nilphamari	8.80± 1.61n-p	77.35 ± 0.26cd	Decrease
AF13	Rangpur	12.13± 1.29k-o	68.77 ± 0.66ij	Decrease
AF11	Rangpur	8.70± 1.29n-p	77.60 ± 0.33cd	Decrease
AF44	Rangpur	3.34 ± 1.64q	91.40 ± 0.75a	Decrease
AF191	Lalmonirhat	3.42 ± 1.48q	91.19 ± 0.38a	Decrease
AF67	Gaibandha	27.25 ±1.31g	29.86 ± 0.95p	Decrease
AF71	Bogura	2.97 ± 1.11q	92.35 ± 0.44a	Decrease

AF75	Bogura	3.10 ± 0.56q	92.03 ± 0.57a	Decrease
AF173	Bogura	9.34 ± 0.45m-p	75.96 ± 1.31c-f	Decrease
AF79	Bogura	13.07± 0.25k-n	66.36 ± 0.83jk	Decrease
AF72	Bogura	39.91± 0.98de	2.73 ± 1.09u	Increase
AF73	Bogura	43.89± 0.24cd	12.98 ± 0.78s	Increase
AF76	Bogura	49.00±0.58b	26.12 ± 0.73q	Increase
AF74	Bogura	8.66± 1.00n-p	77.70 ± 0.96cd	Decrease
AF165	Natore	37.83 ± 0.75ef	2.63± 0.48u	Decrease
AF166	Natore	22.33 ± 0.14h	42.52 ± 0.39o	Decrease
AF185	Natore	45.65± 1.26bc	17.51 ± 1.4r	Increase
AF90	Natore	8.96± 1.18m-p	76.94 ± 0.66cd	Decrease
AF105	Jashore	35.31 ± 1.14f	9.12 ± 1.78t	Decrease
AF102	Jashore	15.52 ± 0.99i-k	60.05 ± 1.22l	Decrease
AF85	Jashore	19.36 ± 1.23hi	50.16 ± 1.33n	Decrease
AF101_3	Jashore	9.72± 1.26mn	74.99 ± 0.19d-g	Decrease
AF101_2	Jashore	40.08±1.00de	3.17 ± 1.77u	Increase
AF202	Chuadanga	5.66 ±2.00pq	85.43 ± 0.25b	Decrease
AF119	Chuadanga	11.43±2.00k-o	70.58 ± 0.49hi	Decrease
AF212	Cumilla	10.78±2.00l-o	72.25 ± 0.81gh	Decrease
Level of Significance		***	***	
CV		173.39	173.21	
LSD		0.44	0.30	
P value		0.00	0.00	

'***' indicate 1% Level of Significance. The *P*-values displayed were obtained from ANOVA and the MSDs (Minimum significant difference) were obtained by Tukey's test. Data were analysed by MINITAB statistical software (version MINITAB-19). Each value represents the mean of three replication. In a column, mean ± SD and figures bearing uncommon letters differ significantly.

608 **Table 4.** Efficacy of atoxigenic *A. flavus* isolates in reducing aflatoxin concentration in inoculated maize cob in net
609 house condition

Isolate no	Area	Total aflatoxin (ng/g)	Reduction of % total aflatoxin	Comments
Control		38.85	0.00	Nill
AF6	Panchagarh	$2.57 \pm 0.28^{i\text{-}k}$	$93.38 \pm 0.38^{a\text{-}d}$	Decrease
AF10	Panchagarh	$2.86 \pm 0.39^{h\text{-}k}$	$92.64 \pm 0.25^{b\text{-}e}$	Decrease
AF176	Panchagarh	$5.38 \pm 0.41^{c\text{-}g}$	86.15 ± 0.38^{mn}	Decrease
AF02	Panchagarh	1.78 ± 0.08^{k}	95.42 ± 0.77^{a}	Decrease
AF01	Panchagarh	12.78 ± 0.56^{a}	67.10 ± 0.28^{q}	Decrease
AF04	Panchagarh	$2.47 \pm 0.74^{i\text{-}k}$	$93.64 \pm 0.49^{a\text{-}c}$	Decrease
AF03	Panchagarh	$4.52 \pm 0.57^{d\text{-}i}$	$88.37 \pm 1.09^{j\text{-}m}$	Decrease
AF07	Panchagarh	$5.4 \pm 1.02^{c\text{-}f}$	86.10 ± 0.46^{mn}	Decrease
AF5	Panchagarh	$2.59 \pm 0.63^{i\text{-}k}$	$93.33 \pm 0.99^{a\text{-}d}$	Decrease
AF168	Panchagarh	$4.6 \pm 0.55^{d\text{-}i}$	$88.16 \pm 0.09^{k\text{-}m}$	Decrease
AF153	Thakurgaon	$3.81 \pm 0.09^{e\text{-}k}$	$90.19 \pm 0.17^{f\text{-}k}$	Decrease
AF14	Thakurgaon	$2.47 \pm 0.29^{i\text{-}k}$	$93.64 \pm 0.55^{a\text{-}c}$	Decrease
AF151	Thakurgaon	7.46 ± 0.67^{c}	80.80 ± 1.32^{o}	Decrease
AF152	Thakurgaon	$4.38 \pm 0.46^{d\text{-}j}$	$88.73 \pm 0.91^{h\text{-}l}$	Decrease
AF31_3	Dinajpur	$3.61 \pm 0.19^{fg\text{-}k}$	$90.71 \pm 0.49^{e\text{-}k}$	Decrease
AF34	Dinajpur	$4.05 \pm 0.37^{d\text{-}j}$	$89.58 \pm 0.55^{g\text{-}l}$	Decrease
AF27	Dinajpur	$3.39 \pm 0.29^{f\text{-}k}$	$91.27 \pm 0.36^{c\text{-}g}$	Decrease
AF23	Dinajpur	$3.14 \pm 0.36^{h\text{-}k}$	$91.92 \pm 0.44^{b\text{-}g}$	Decrease
AF31_2	Nilphamari	$2.84 \pm 0.10^{g\text{-}k}$	$92.69 \pm 0.73^{b\text{-}e}$	Decrease
AF13	Rangpur	$2.86 \pm 0.31^{h\text{-}k}$	$92.64 \pm 0.09^{b\text{-}e}$	Decrease
AF11	Rangpur	$4.5 \pm 1.34^{d\text{-}i}$	$88.42 \pm 1.21^{j\text{-}m}$	Decrease
AF44	Rangpur	$4.3 \pm 1.23^{d\text{-}j}$	$88.93 \pm 0.51^{g\text{-}l}$	Decrease
AF191	Lalmonirhat	$2.45 \pm 0.89^{i\text{-}k}$	$93.69 \pm 0.11^{a\text{-}c}$	Decrease
AF67	Gaibandha	$4.6 \pm 1.39^{d\text{-}i}$	$88.16 \pm 1.11^{k\text{-}m}$	Decrease
AF71	Bogura	$5.38 \pm 1.56^{c\text{-}g}$	86.15 ± 1.44^{mn}	Decrease
AF75	Bogura	$3.78 \pm 0.15^{e\text{-}k}$	$90.27 \pm 0.88^{e\text{-}k}$	Decrease
AF173	Bogura	$4.34 \pm 0.22^{d\text{-}j}$	$88.83 \pm 0.75^{h\text{-}l}$	Decrease
AF79	Bogura	$4.62 \pm 0.47^{d\text{-}i}$	$88.11 \pm 0.49^{j\text{-}m}$	Decrease
AF72	Bogura	$3.51 \pm 0.55^{f\text{-}k}$	$90.97 \pm 0.62^{d\text{-}i}$	Decrease
AF73	Bogura	$3.08 \pm 0.12^{h\text{-}k}$	$92.07 \pm 0.88^{b\text{-}f}$	Decrease
AF76	Bogura	$4.48 \pm 0.35^{d\text{-}i}$	$88.47 \pm 1.25^{j\text{-}m}$	Decrease
AF74	Bogura	1.81 ± 0.10^{k}	95.34 ± 0.6^{a}	Decrease
AF165	Natore	9.85 ± 1.56^{b}	74.65 ± 1.24^{p}	Decrease
AF166	Natore	6.03 ± 0.88^{cd}	84.48 ± 0.51^{n}	Decrease
AF185	Natore	$4.58 \pm 1.0^{d\text{-}i}$	$88.21 \pm 0.29^{k\text{-}m}$	Decrease
AF90	Natore	$5.85 \pm 0.77^{c\text{-}e}$	84.94 ± 1.37^{n}	Decrease
AF105	Jashore	$4.81 \pm 0.35^{d\text{-}h}$	87.62 ± 0.73^{lm}	Decrease
AF102	Jashore	$2.52 \pm 0.25^{i\text{-}k}$	$93.51 \pm 0.09^{a\text{-}c}$	Decrease
AF85	Jashore	$3.31 \pm 0.34^{e\text{-}k}$	$91.48 \pm 0.10^{c\text{-}g}$	Decrease
AF101_3	Jashore	$3.59 \pm 0.06^{f\text{-}k}$	$90.76 \pm 0.09^{e\text{-}j}$	Decrease

AF101_2	Jashore	$3.92 \pm 0.08^{d\text{-}k}$	$89.91 \pm 1.00^{f\text{-}l}$	Decrease
AF202	Chuadanga	2.22 ± 0.05^{jk}	94.29 ± 0.05^{ab}	Decrease
AF119	Chuadanga	$3.96 \pm 0.41^{d\text{-}k}$	$89.81 \pm 0.82^{f\text{-}l}$	Decrease
AF212	Cumilla	$3.92 \pm 0.06^{d\text{-}k}$	$89.91 \pm 0.47^{f\text{-}l}$	Decrease
Level of Significance		***	***	
CV		173.17	173.22	
LSD		0.21	0.23	
P value		0.00	0.00	

610 '***'indicate 1% Level of Significance. The *P*-values displayed were obtained from ANOVA and the MSDs
611 (Minimum significant difference) were obtained by Tukey's test. Data were analysed by MINITAB statistical software
612 (version MINITAB-19). Each value represents the mean of three replication. In a column, mean ± SD were added and
613 figures bearing uncommon letters differ significantly

614

Figures

Figure 1.

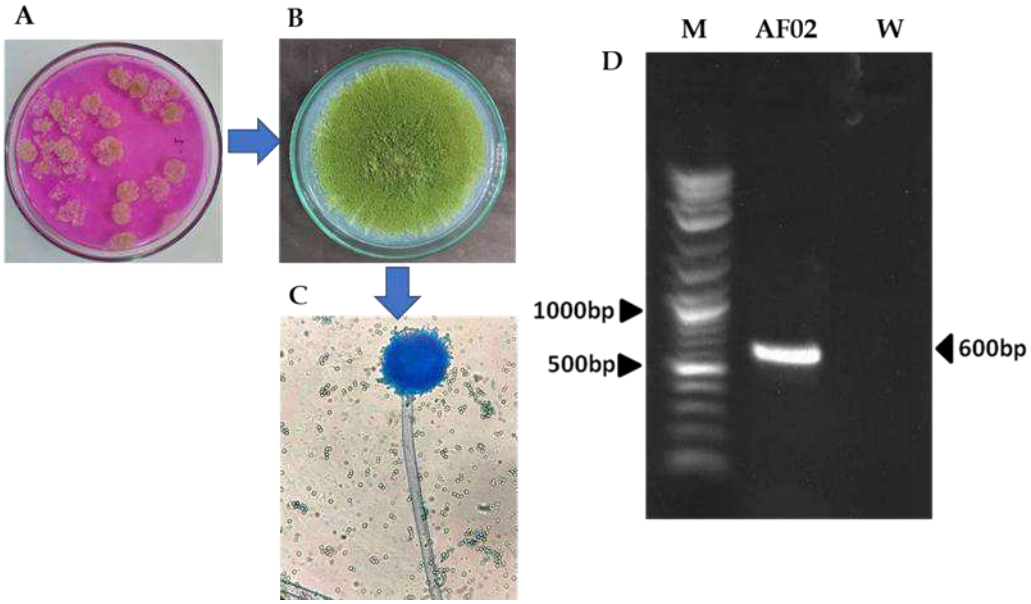


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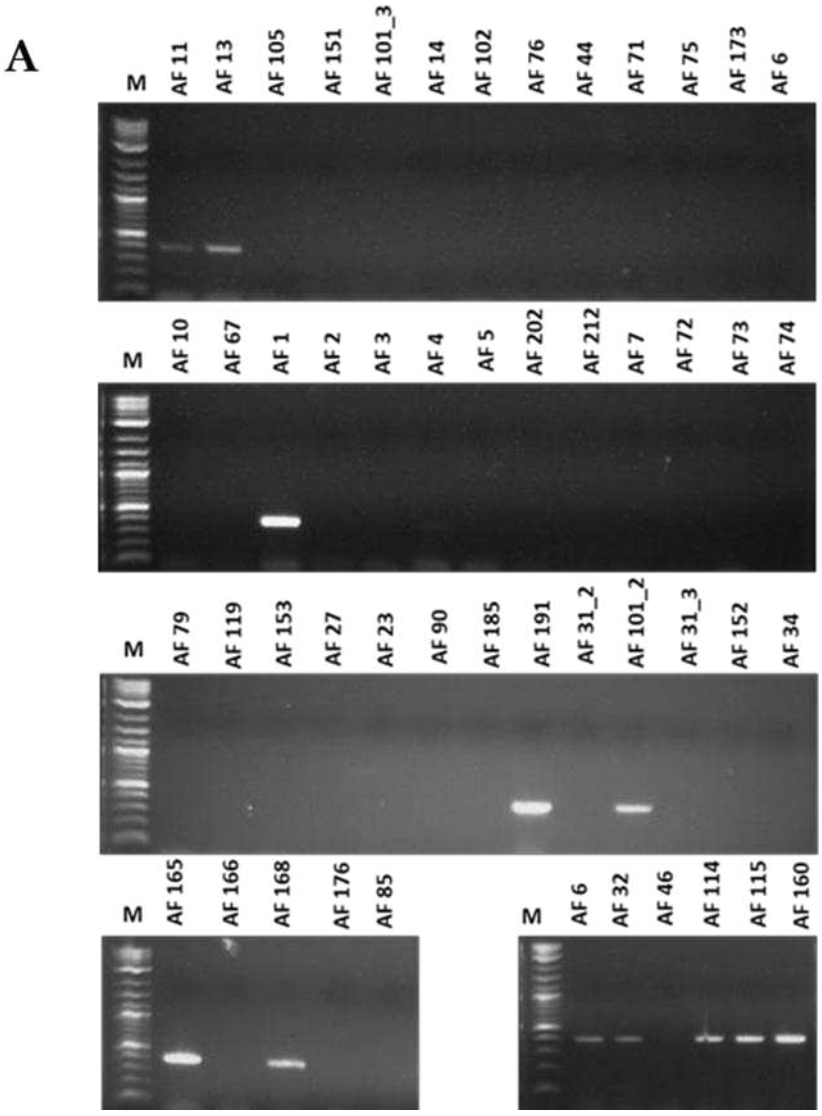

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AF 101_3
AF 14
AF 102
AF 76
AF 44
AF 71
AF 75
AF 173
AF 6
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AF 10
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AF 1
AF 2
AF 3
AF 4
AF 5
AF 202
AF 212
AF 7
AF 72
AF 73
AF 74
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AF 79
AF 119
AF 153
AF 27
AF 23
AF 90
AF 185
AF 191
AF 31_2
AF 101_2
AF 31_3
AF 152
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AF 6
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AF 115
AF 160

Figure 2. B)

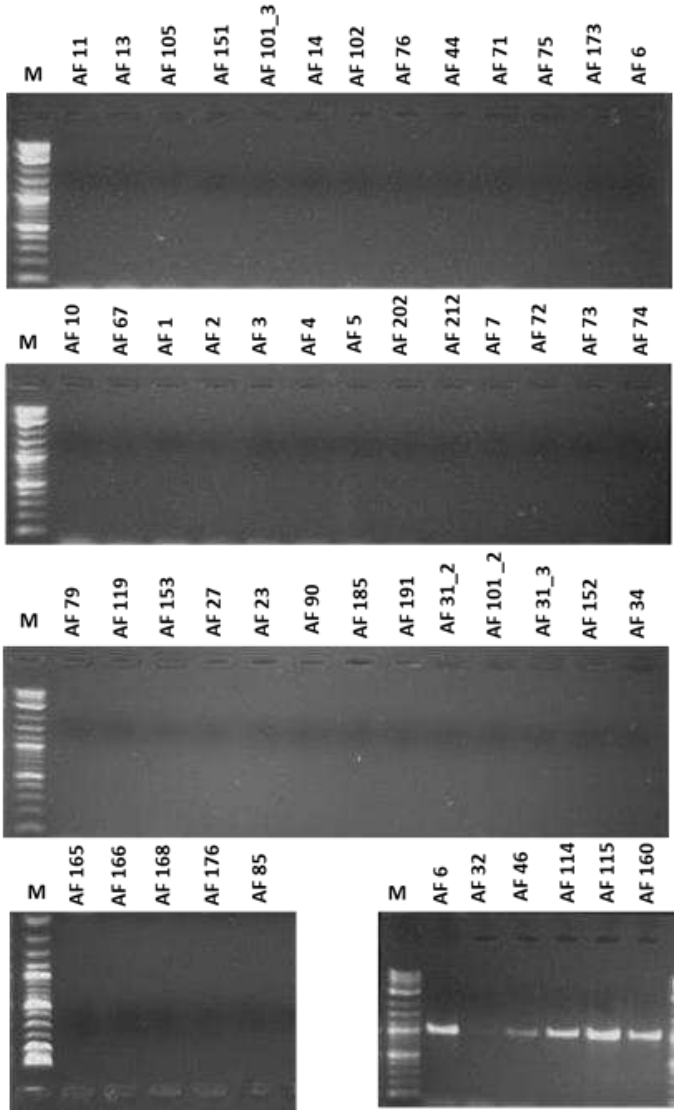
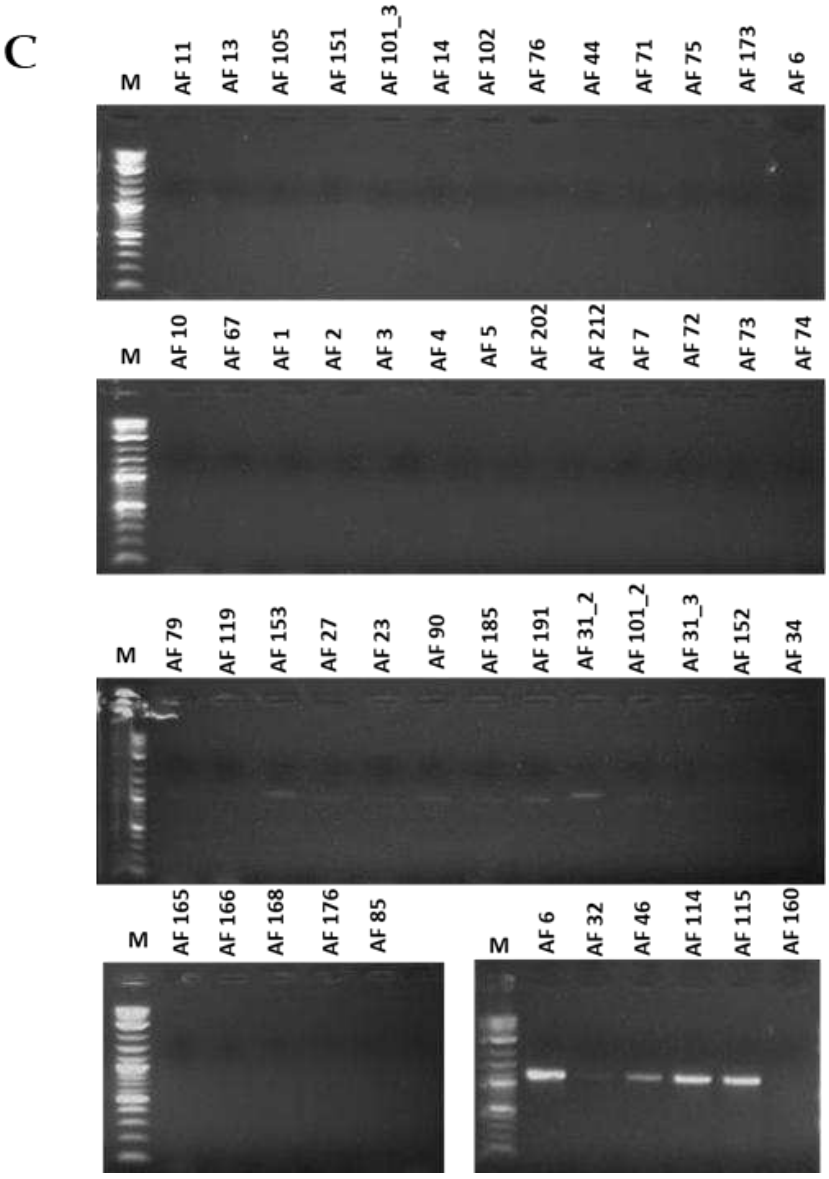

B
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Figure 2. C)


C
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AF 11
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AF 151
AF 101_3
AF 14
AF 102
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AF 44
AF 71
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AF 173
AF 6
M
AF 10
AF 67
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Supplementary Materials

Supplementary Figures

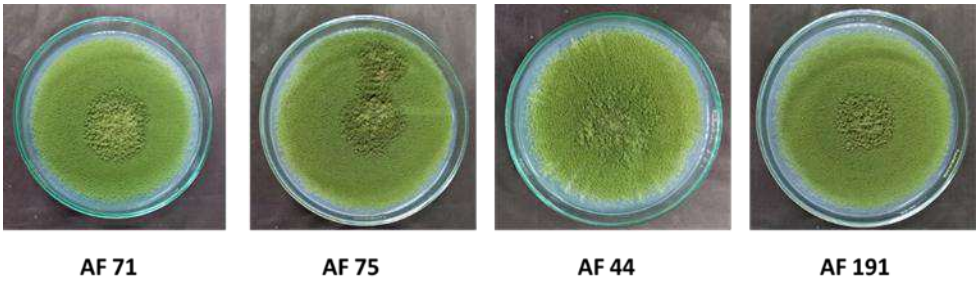
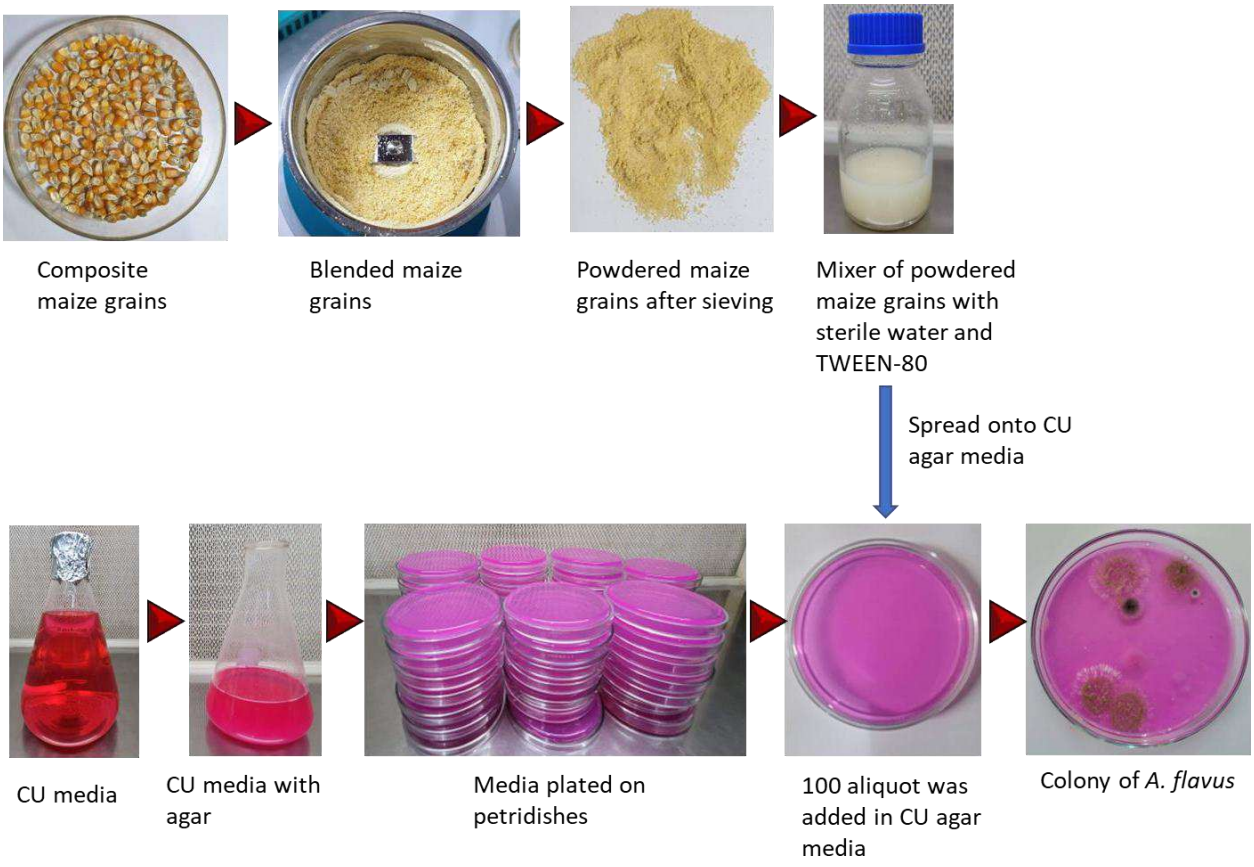


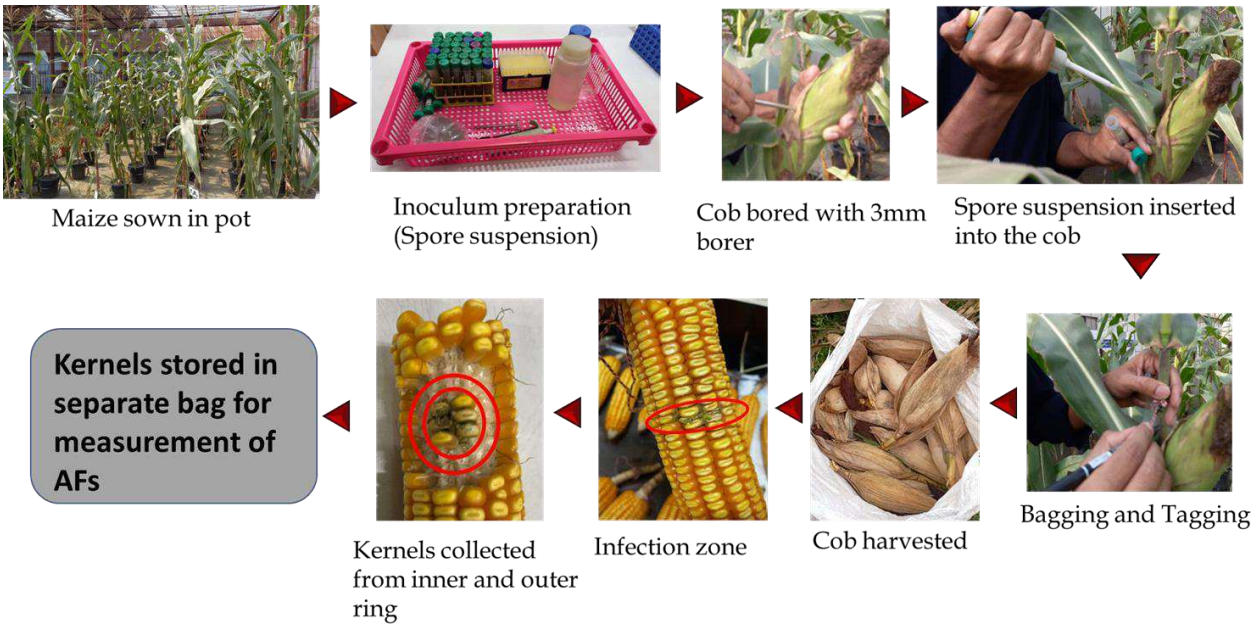
Figure S1. Pure culture of four representative *A. flavus* isolates on PDA medium



634

635 **Figure S2.** Steps for isolation of *A. flavus* isolates on CU (Clean Up) agar medium

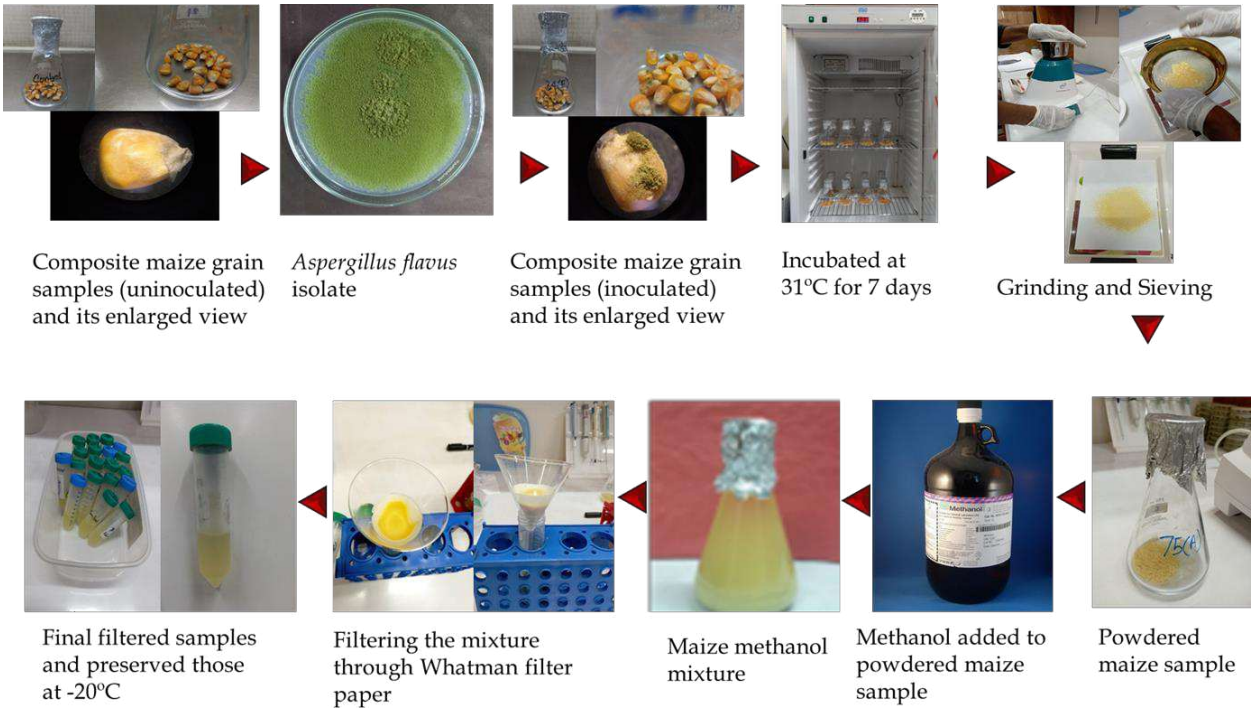
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637

638 **Figure S3.** Artificial inoculation with *A. flavus* isolates at mid-silking stage and collection maize grain samples for
639 total aflatoxin measurement

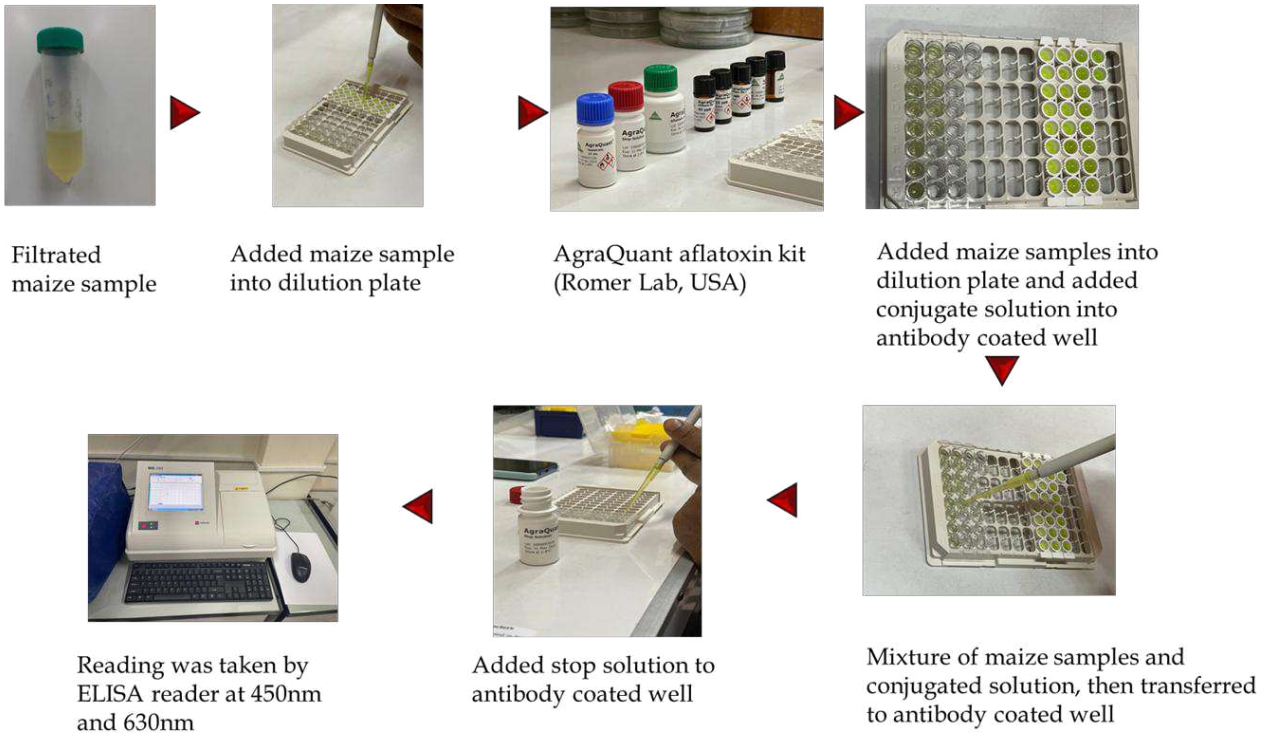
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641

642 **Figure S4.** Steps of sample preparation for the detection of total aflatoxin from 10g of stored maize grains

643



644

645 **Figure S5.** Steps of assay protocol for the detection of total aflatoxin in maize grains inoculated with *A. flavus*
646 isolates using AgraQuant aflatoxin kit (Romer Lab, USA)