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CATEGORIZATION OF *ASPERGILLUS FLAVUS* AND *ASPERGILLUS PARASITICUS* ISOLATES OF STORED WHEAT GRAINS IN TO AFLATOXINOGENICS AND NON-AFLATOXINOGENICS

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Abstract

The objective of this study was to categorize 157 *Aspergillus flavus* (AF1-AF157) and 36 *A. parasiticus* (AP1-AP36) strains isolated from stored wheat grains from three provinces of Pakistan viz., Punjab, Sindh and NWFP, into aflatoxigenic and non-aflatoxigenic ones by cultural as well as PCR methods. None of the isolates produced aflatoxin except the AP4 isolate. The positive control in all the batches of *A. flavus* and *A. parasiticus* isolates produced maximum aflatoxin in grains at 16% moisture and 25ºC temperature in a laboratory experiment of this study. The accuracy of cultural tests ELIZA assessed by percent recovery of the toxin from positive controls, spiked samples and spiked controls ranged from 98–100%. An assay based on multiplex PCR was applied for the detection of four genes located at different loci coding enzymes in the aflatoxin biosynthetic pathway of *A. flavus* and *A. parasiticus* strains. These are AflR, reported as regulatory gene and functions as a transcription activator, others are structural genes named according to their substrates Nor 1 (norsolorinic acid), Ver 1(Versicolorin) and Omt (O methylosterigmatocystin). Recovery of these four genes in the DNA template of known Aflatoxinogenic *A. flavus* strains in every experimental trial of present study as a positive control showed the accurate PCR experimentation. None of the *A. flavus* and 35 *A. parasiticus* (AP1-AP3 and AP5-AP36) isolates produced aflatoxin in both the flask and storage experiments. Similarly all these strains did not exhibit presence of all the four genes in the PCRs of the extracted DNA at one time. All the *A. flavus* isolates on the basis of their gene pattern were grouped into 11 groups and *A. parasiticus* isolates into 9 groups. Presence of all four genes was detected only in one aflatoxinogenic isolate of *A. parasiticus* (AP4). The comparison of the cultural and PCR methods showed good agreement, as results of both the methods exclusively matched each other. All the non Aflatoxinogenic isolates of this study showed biocontrol activity against known Aflatoxinogenic *A. flavus* isolate during an *in vitro* laboratory experiment.

Introduction

Aflatoxins are carcinogenic secondary metabolites produced primarily by two *Aspergillus* species viz., *A. flavus* and *A. parasiticus*. Toxigenic moulds may invade agricultural products during plant growth, during harvest and afterwards in storage (Silva *et al.*, 2000; Tancino *et al.*, 2001). Due to the toxic and carcinogenic properties of aflatoxins, there is a need to develop reliable methods to detect the presence of aflatoxigenic *Aspergilli* in contaminated foods and feed. Not all *Aspergillus* strains are able to produce aflatoxins and this prompts the adoption of multiple screening techniques to ascertain the real toxigenic potential of contaminating molds.

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Conventional methods with developed techniques are used to distinguish among toxigenic and nontoxigenic isolates in the *A. flavus* group. This involves culturing the fungus in suitable inducing media, extracting aflatoxins with organic solvents, and monitoring their presence by chromatographic and ELIZA techniques (Lin *et al*., 1980; Yang *et al*., 2004). Hamed *et al*., (2004) suggested combined assay of cultural method that reduced the false positives of aflatoxinogenecity to 0% and false negatives to 7%. Chen *et al*., (2002) carried multiplex PCR detection of genes involved in the aflatoxin biosynthetic pathway. In the literature a lot of scientists demonstrated that aflatoxinogenic strains could be differentiated from non aflatoxigenic by real-time RT-PCR method (Doohan *et al*., 1999; Sweeney *et al*., 2000; Mayer *et al*., 2003; Barbera *et al*., 2004). Tran-Dinh *et al*., (1999) examined the genetic relationship between toxigenic and nontoxigenic isolates using RAPD analysis *A. flavus* isolates.

The aim of this study was to categorise 157 *Aspergillus flavus* (AF1-AF157) and 36 *Aspergillus parasiticus* (AP1-AP36) isolates of stored wheat grains in to aflatoxigenic and non-aflatoxigenic ones. During cultural methods isolates were checked in sterilized and Aflatoxin free wheat and rice grains (used as natural media) and toxin level was tested by ELIZA technique. In the molecular studies sets of primers matching a series of key genes in the Aflatoxin biosynthetic pathway were designed and used in analyzing a collection of well-characterized isolates of *A. flavus* and *A. parasiticus* investigating the genetic variability of *A. flavus* and *A. parasiticus* population in Pakistan.

Materials and Methods

Preservation of pure cultures of *A. flavus* and *A. parasiticus*: Isolated pure cultures of *A. flavus* and *A. parasiticus* were preserved on Czapek's agar slants and in the silica gel at 4 ± 0.02°C. The purity of preserved cultures was observed twice in a month by dissolving a loop full of culture from slants and some crystals of silica gel preserved tubes of each isolate in one milliliter sterilized water and adjusting the same spore concentration (10^3 spores per ml) by Haemocytometer for all the picked cultures and then was plated on the Czapek's agar medium. Plates were incubated at 25± 0.02°C and after 5–6 days colonies of pure culture and of contaminants were noted and calculated. A comparison between the two preserved methods for isolated cultures purification was determined.

Laboratory experiment to determine toxin producing ability of *A. flavus* and *A. parasiticus* isolates: *A. flavus* strains collected / isolated from samples were refreshed on AFPA medium and these strains were tested for their toxin producing ability on the sterilized rice and wheat (negative for Aflatoxin as determined by ELIZA) as a solid substrate at 16% moisture level. *A. flavus* and *A. parasiticus* isolates, each in three replicates were individually inoculated with 1ml of a spore suspension (10^3 spores /ml) in 15g sterilized rice in 250ml flasks separately. Each sample was in triplicate having uninoculated (only media) and inoculated (with Aflatoxinogenic *A. flavus* obtained from Canadian culture powder) controls. The inoculated flasks were fixed in thermo thermostatically controlled water bath shaker at 25+1ºC for eight days. Same strains were also treated under same conditions in sterilized wheat. ELIZA test was performed on 8th day to check the toxins produced in the inoculated media and also in the uninoculated control.

Laboratory study to evaluate effect of storage conditions on *A. flavus A. parasiticus* growth and contamination of wheat grain: It was a CRD experiment with following treatments:

Treatments: 9
Moisture level: 3 (9, 12 and 14%)
Temperature: 3 (25, 30 and 35 ºC)
Number of replicates: 3

Wheat grains to be used for experimentation were cleaned manually to remove extraneous matter and were fumigated using Phosphene gas to eliminate possible insect contamination, and then checked for aflatoxin contamination using Neogen ELIZA kit and also checked for *A. flavus* and *A. parasiticus* growth on AFPA medium. The grains without aflatoxin and having none of the fungi, was split into three parts and tempered by the addition of appropriate amount of sterilized distilled water to raise water for required tempering to achieve three moisture levels 9, 12 and 14% by the formula:

$$\text{Water to be added in ml} = \frac{\text{Grams of wheat grains X 100- OM}}{\text{100-DM}} \text{ X 100}$$

where:
OM= Original moisture content of sample
DM= Desired moisture content of sample

The wheat was conditioned in batches by placing each one in airtight sterilized glass jars. The added water was mixed for 5 minutes and allowed to rest for 24 hours to distribute the moisture evenly (Hall, 1970). A composite of spores in which each Vehari *A. flavus* isolates at 10^3 spores /ml concentration counted by haemocytometer was mixed and inoculated in fumigated and Aflatoxin free wheat grains. Wheat grains splitted for 9 treatments under different temperature conditions (25, 30 and 35ºC) and moisture conditions (9, 12 and 14%) and were stored for 6 months from 23.11.04 to 25.04.05 with three replicates of each treatment in Pervical incubators. Un-inoculated and inoculated (with Aflatoxin producing positive culture) controls for each treatment were also maintained. The tempered moisture contents (9, 12 and 14%) at particular relative humidity (30, 60 and 80%) were maintained by the addition of sterilized silica gel in the sterilized muslin cloth quantified by James & Johnson (1970) method. Sub sampling at (one month) intervals was carried out and then these sub samples were analysed for their inoculated *A. flavus* isolates growth status and their Aflatoxin production.

Same experiment was designed for *A. flavus* isolates (in composite) of Sahiwal, Multan, Pakpattan, Gujarkhan, Muzzafargarh, Bahawalpur, Gujranwala, Karachi, Nawabshah, Mirpurkhas and Nowshehra for the duration of 03.06.05 to 01.11.05. The tempered moisture contents (9, 12 and 14%) of inoculated grains at particular relative humidity (30, 60 and 80%) and temperature (25, 30 and 35ºC) were maintained in three Pervical growth chambers having their display systems of maintained temperature, moisture content and relative humidity.

Third time same experiment was designed to check the toxin production ability of *A. parasiticus* strains composite isolated from Pakpattan, Muzzafargarh, Lahore, Nawabshah and Naushehro Feroz for the duration of 01.12.05 to 01.05.06.

Molecular identification of aflatoxinogenic and non-aflatoxinogenic isolates: Extraction of genomic DNA of isolated strains was carried out by The E.Z.N.A. ® Fungal DNA Mini Kits after freezing at – 80ºC and pulverizing with a pestle and mortar. Quantification and Quality Assessment of DNA extracted from *A. flavus* and *A. parasiticus* strains was done by spectrophotometry.

The isolated DNA was amplified in Biometra PCR instrument. The Primer sequencing was confirmed with the assistance of BLAST.

nor1, 5'-ACC-GCTACGCCGGCACTCTCGGCAC-3',
nor2, 5'-TTGGCCGCCAGCTTCGACACTCCG-3'enclosing a fragment of 400 bp
ver1, 5'-GCCGCAGGCCGCGGAGAAAGTGGT-3'
ver2, 5'-GGGGATATACTCCCGCGACACAGCC-3', enclosing a fragment of 600 bp
omt1, 5'-GTGGACGGACCTAGTCCGACATCAC-3',
omt2, 5'-GTC-GGCGCCACGCACTGGGTTGGGG-3'enclosing a fragment of 797 bp
aflR1, 5'-TATCTCCCCCCGGGCATCTCCCGG-3',
aflR2, 5'-CCGTCAGACAGCCACTGGACA-CGG-3',, enclosing a fragment of 1000 bp

PCR assays were performed in 25µl of a reaction mixture that contained (Buffer, 2.5 µl, MgCl2, 1.5µl, dntps, 0.5µl, Taq polymerase, 0.5µl, Primer nor1 (reverse), 0.5µl Primer nor1 (Forward), 0.5µl Primer ver1 (reverse), 0.5µl Primer ver1 (Forward), 0.5µl Primer aflR (reverse), 0.5µl Primer aflR (Forward), 0.5µl Primer omt1 (reverse), 0.5µl Primer omt (Forward), 0.5µl Template DNA, 1.5µl, volume of master mix., 10. 5µl and Deionized water, 14.5 µl was added to make the volume up to 25µl. PCR amplification conditions were 5 min for denaturation at 96°C, followed by 35 cycles: 95°C, 1 min; 65°C, 30 seconds; 72°C, 30 seconds for the first cycle; and 94°C, 30 seconds; variable (56 -62°C), 30 seconds; 72°C, 30 seconds for the next 34 cycles. Twelve micro liter of the PCR products with positive and negative controls was loaded in the gel wells after mixing with appropriate amount of 6.7 µl of 6X loading dye. 7.5 µl of 100 bp ladder mixed in 6X loading orange dye were also loaded. PCR products were electrophoresed in a 1.2% agarose gel prepared in 1X TBE stained with ethidium bromide (1.5 µg/ml) for 2 to 3 h. DNA bands were visualized on a UV Tran illuminator at a wave length of 540nm. Analyzed strains were grouped according to the presence and absence of genes.

***In vitro* study to check bio control activity of present study atoxinogenic isolates of *A. flavus* and *A. parasiticus* against aflatoxinogenecity of *A. flavus* and *A. parasiticus*:** In this laboratory experiment spores of Canadian culture of Aflatoxinogenic *A. flavus* isolate at 10^3 spores per milliliter concentration was inoculated in 500 ml flask having hundred grams Aflatoxin free and sterilized wheat grains. Similarly in another 500 ml flask spores of Aflatoxinogenic *A. parasiticus* isolate AP4 of present study at same concentration were inoculated in hundred grams wheat. In second treatment a composite of all the non Aflatoxinogenic *A. flavus* isolates (AF1-AF157) at 10^3 spores per milliliter concentration of each were inoculated in two separate flasks each having hundred grams sterilized wheat. One flask remained as such and in other flask Canadian Aflatoxinogenic isolate of *A. flavus* at 10^3 spores per milliliter concentration was inoculated. Two other flasks with hundred grams sterilized and Aflatoxin free wheat were inoculated with non Aflatoxinogenic *A. parasiticus* isolates (AP1-AP3 and AP5-AP36) of present study at 10^3 spores per milliliter concentration. Only in one flask *A. parasiticus* isolate AP4 of present study at 10^3 spores per milliliter concentration was also inoculated. All these treatments were maintained at controlled conditions of 16% moisture contents with 85% relative humidity and 25ºC temperature for eight days. On eight day, extracts of all the inoculated wheat were analyzed for their Aflatoxin concentration. Each treatment was maintained in three replicates.

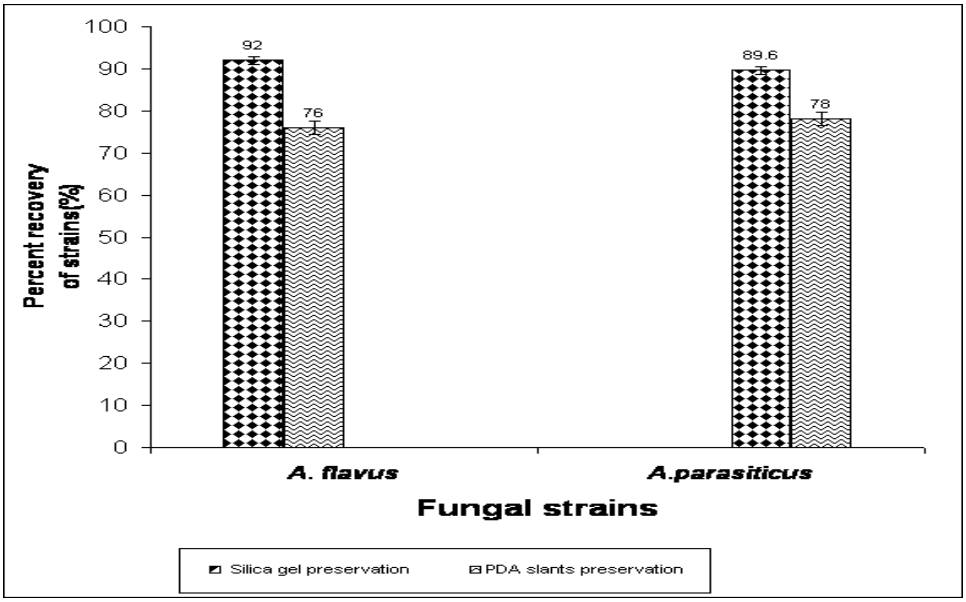


Fig. 1. Comparison of preservation methods of isolated fungal strains.
Each bar value representative of *A. flavus* is the mean of three replicates x 157 *A. flavus* isolates. Each bar value representative of *A. parasiticus*.is the mean of three replicates x 36 *A. parasiticus* isolates. Error bars represent the ± SE.

Results

All the preserved cultures used in this study showed more percent recovery in silica gel medium as compared to the PDA slants (Fig. 1). None of the AF1-AF157 isolates of *A. flavus* produced Aflatoxin neither in the sterilized wheat nor in rice moistened with deionized water flask experiment nor in the storage experiment (Figs. 2 & 4; Tables 1 & 2). The PCR analysis also supported these findings as none of these isolates exhibited the presence of all the four genes (Figs. 6 & 7). On the basis of genes presence, isolates were divided in to different groups. Isolates with same patterns for the presence or absence of four genes in this study were grouped in one group as shown in Table 4., while to check the ELIZA test performance in all experiments positive standards and controls with spiked standards and samples that produced the toxin was done by percent recovery and found more than 98% as in the Figs. 3 and 5. The sterilized wheat grains of storage experiment were 100% inoculated as checked through out the storage in sub samples monthly drawn from whole sample (data not shown). Among 36 *A. parasiticus* isolates only AP4 isolate produced the toxin in shake flask experiment as shown in Figs. 4 and 5. This is the only strain that showed the presence of all the four genes necessary for the toxin production as shown in the Fig. 8. On the basis of varying banding patterns *A. flavus* and *A. parasiticus* isolates were divided into different groups (Tables 4 & 5).

In vitro studies of Non Aflatoxinogenic isolates of 157 *A. flavus* AF1-AF157 and 35 *A. parasiticus* isolates of AP1-AP3 and AP5-AP36 revealed that when inocula of these isolates was added in the Aflatoxinogenic *A. flavus* Canadian culture and Aflatoxinogenic *A. parasiticus* AP4 isolate of present research they minimized the Aflatoxin biosynthesis of Aflatoxinogenic isolates (Fig. 9).

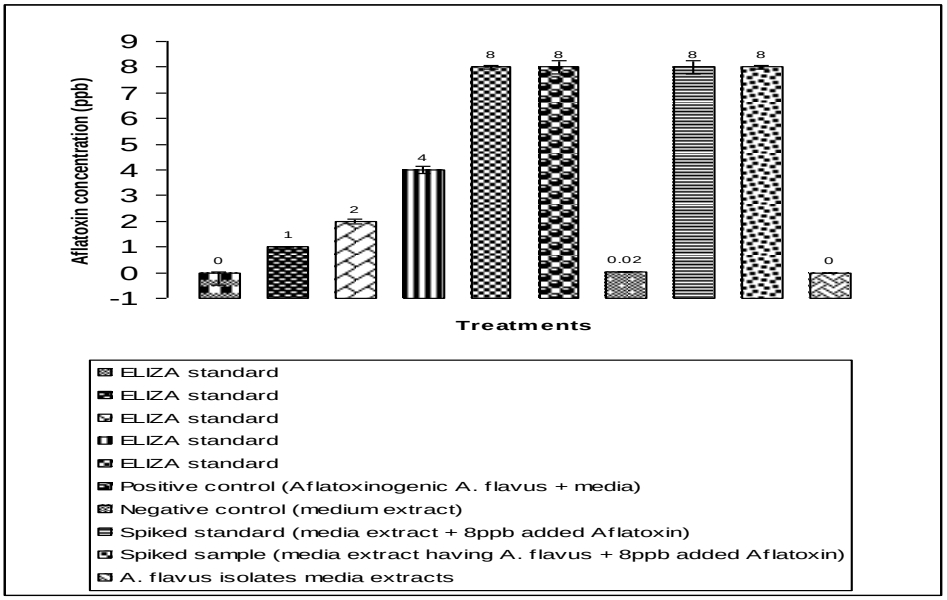


Fig. 2. *In vitro* study of Aflatoxin production ability of *A. flavus* strains in sterilized and aflatoxin free rice and wheat media.
Each value is the mean of three replicates x 157 isolated *A. flavus* strains. Error bars represent the ± SE.

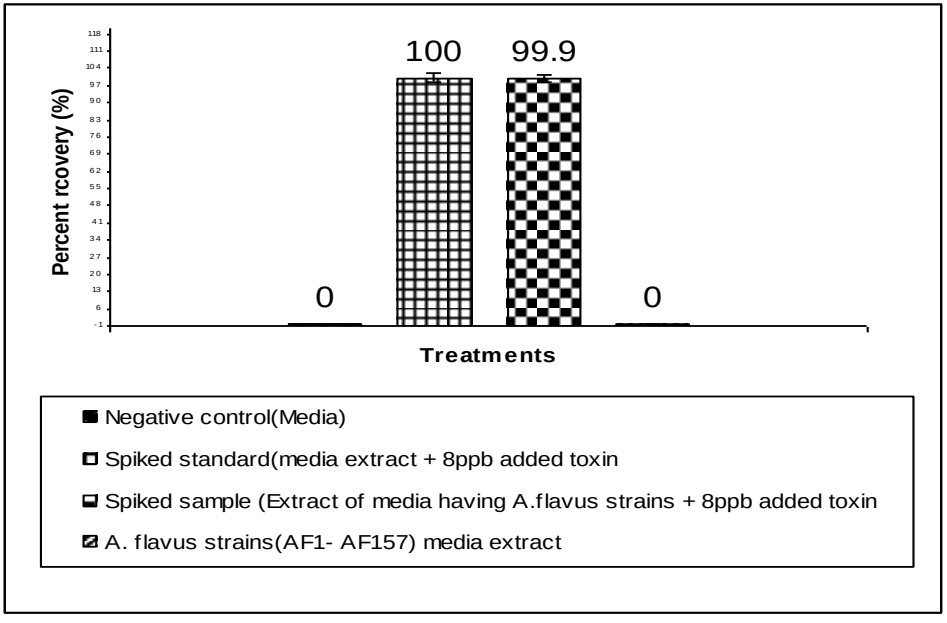


Fig. 3. Percent recovery of Aflatoxin from spiked samples of *A. flavus* in sterilized and Aflatoxin free rice and wheat media.
Each value is the mean of three replicates x 157 isolated *A. flavus* strains. Error bars represent the ± SE.

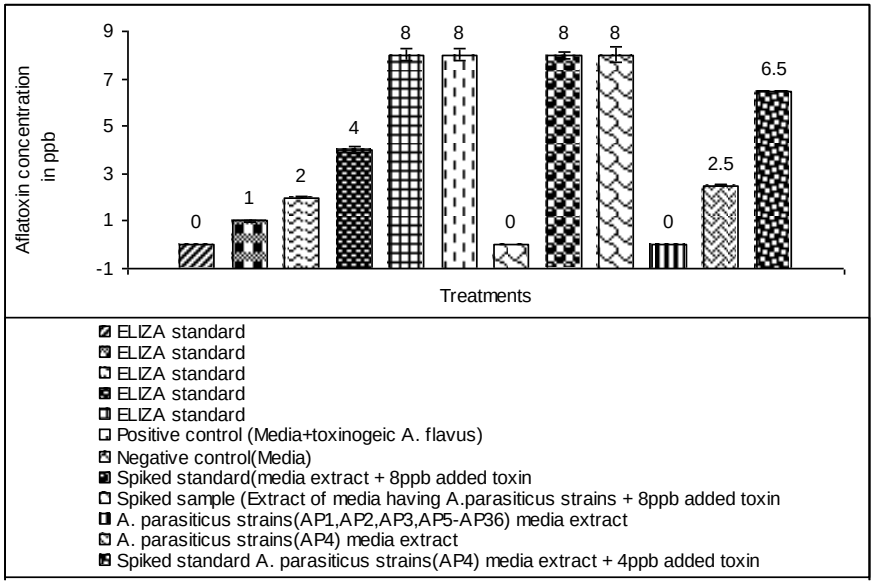


Fig. 4. *In vitro* study to check the Aflatoxin producing ability of *A. parasiticus* in sterilized Aflatoxin free rice and wheat media.
Each value is the mean of three replicates x 36 isolated *A. parasiticus* strains. Error bars represent the ± SE.

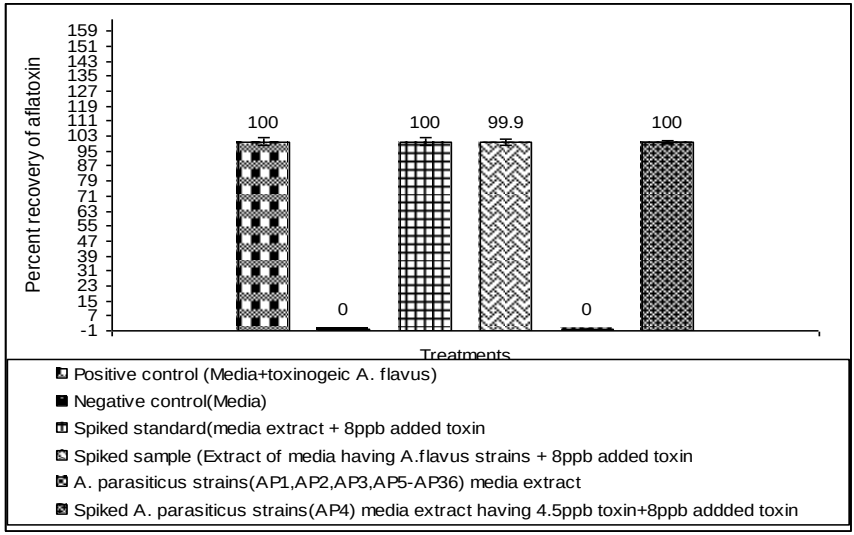


Fig. 5. Percent recovery of Aflatoxin produced by *A. parasiticus* spiked samples in sterilized Aflatoxin free rice and wheat medium.
Each value is the mean of three replicates x 36 isolated *A. parasiticus* strains. Error bars represent the ± SE.
Note: In spiked AP4 sample 4ppb Aflatoxin was spiked in the second repeat of this strain extract, because when 8ppb was spiked it could not be measured due to the standards limitation.

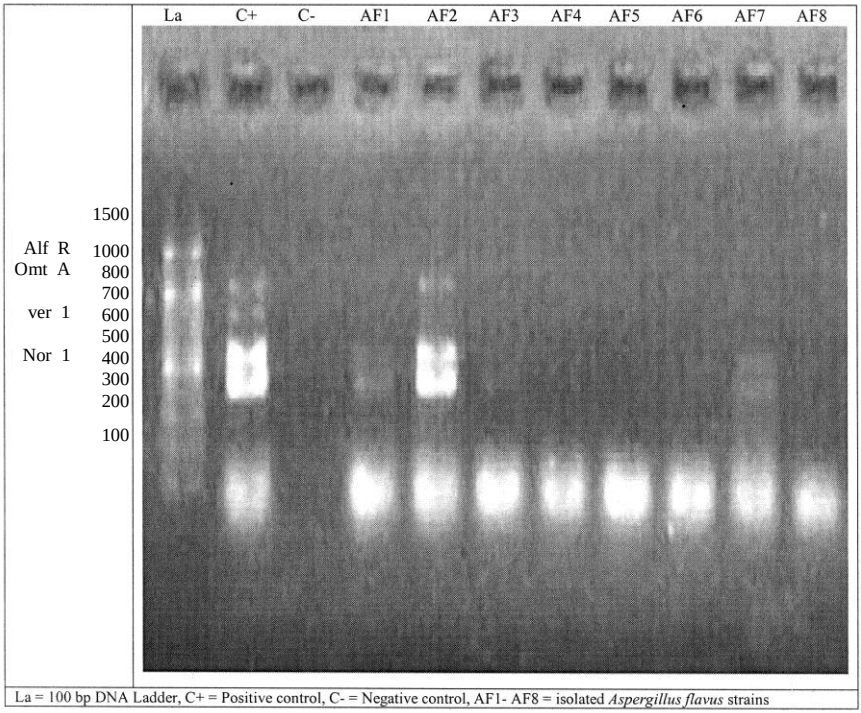


Fig. 6. Bands amplified by the quadruplex PCR of the extracted DNA from the *A. flavus* isolates (AF1-AF8).

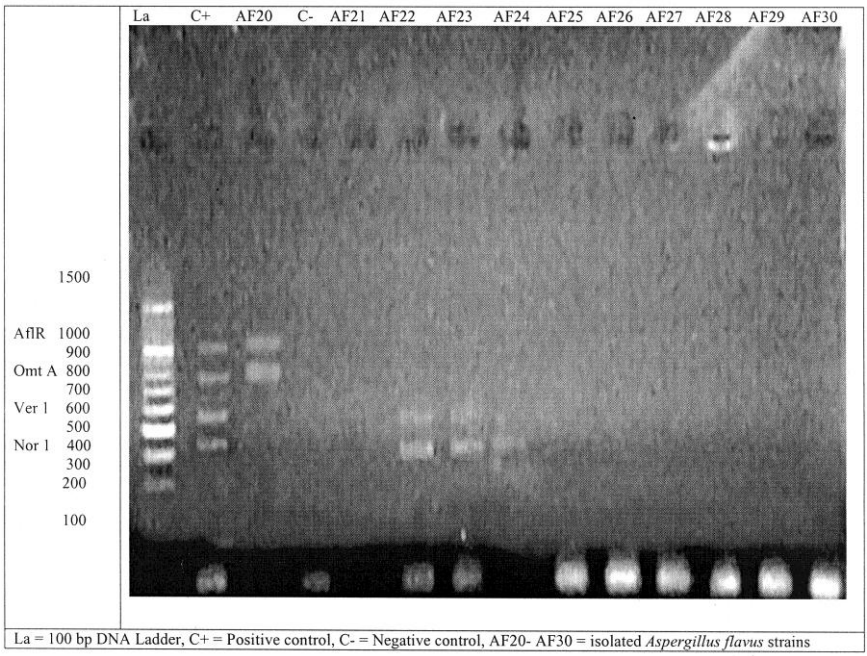
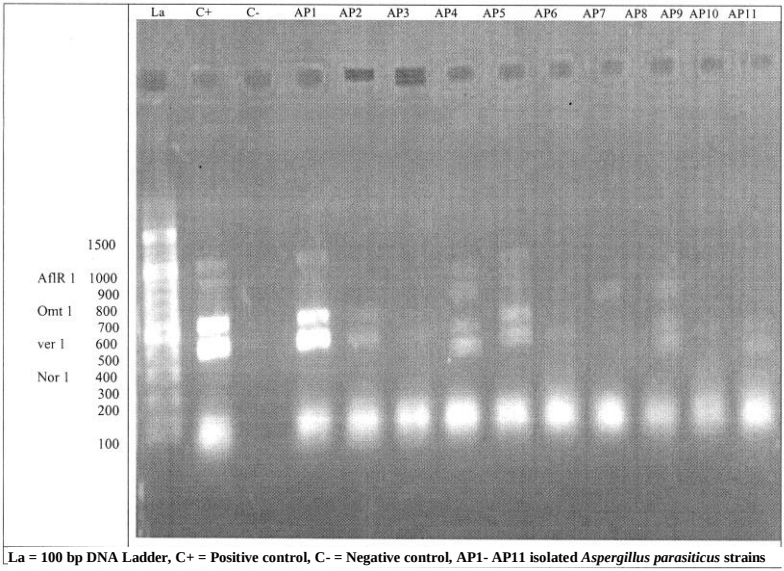


Fig. 7. Bands amplified by the quadruplex PCR of the extracted DNA from the *A. flavus* isolates (AF20-AF30).



La = 100 bp DNA Ladder, C+ = Positive control, C- = Negative control, AP1- AP11 isolated *Aspergillus parasiticus* strains

Fig. 8. Bands amplified by the quadruplex PCR of the extracted DNA from the *A. parasiticus* isolates (AP1-AP11).

Table 5. Grouping of *A. parasiticus* isolates based on genetic biodiversity.

Groups	Percentage of isolates (%)	Isolated strains of *Aspergillus parasiticus*	Molecular genetic sizes			
			AflR 1000	Omt A 800	Ver 1 600	Nor 1 400
A	8.33	AP_1 AP_5 AP_8	+	-	+	+
B	2.78	AP4	+	+	+	+
C	5.56	AP_{18} AP_{19}	-	-	+	-
D	2.78	AP_{29}	-	+	-	+
E	50.00	AP_3 AP_{11} AP_{13} AP_{14} AP_{16} AP_{20} AP_{21} AP_{22} AP_{23} AP_{24} AP_{26} AP_{28} AP_{30} AP_{32} AP_{33} AP_{34} AP_{35} AP_{36}	-	-	-	-
F	2.78	AP_9	-	-	-	+
G	11.11	AP_2 AP_6 AP_{10} AP_{27}	-	-	+	+
H	8.33	AP_{31} AP_7 AP_{25}	-	+	-	-
I	8.33	AP_{12} AP_{15} AP_{17}	-	+	+	+

Each value is the mean of two replicates of each *A. parasiticus* isolate.

As shown in Fig. 9 that Aflatoxinogenic Canadian culture of *A. flavus* when inoculated in wheat substrate at 16% moisture content and 25ºC temperature conditions, 8 ppb Aflatoxin was detected in the wheat substrate extract by the ELIZA kit, but Aflatoxin concentration by this isolate was found 7.4 ppb when an inocula of Non Aflatoxinogenic isolates of 157 *A. flavus* was also incubated with this Canadian culture of *A. flavus.*

Non-aflatoxinogenic 35 isolates of *A. parasiticus* (AP1-AP3 and AP5-AP36) to check their biocontrol activity on aflatoxin production of aflatoxinogenic *A. parasiticus* (AP4) revealed that these isolates minimized the aflatoxin biosynthesis of AP4 as this isolate when inoculated in wheat substrate without non-aflatoxinogenic *A. parasiticus* isolates, 7.4 ppb aflatoxin was found in wheat extract, but with inocula of non-aflatoxinogenic aflatoxin concentration of 6.9 ppb was detected.

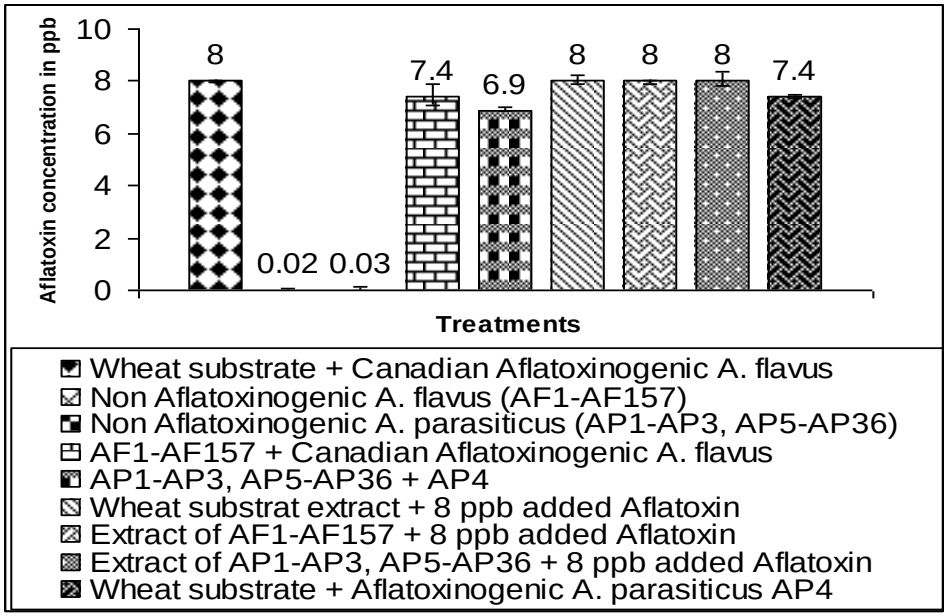


Fig. 9. *In vitro* study of non Aflatoxinogenic *A. flavus* and *A. parasiticus* isolates as biocontrol agents against Aflatoxinogenic *A. flavus* and *A. parasiticus* isolates.
Each bar value is the mean of three replicates of each treatment X values recorded for all analyzed batches. Error bars are the representatives of ±SE of each treatment.

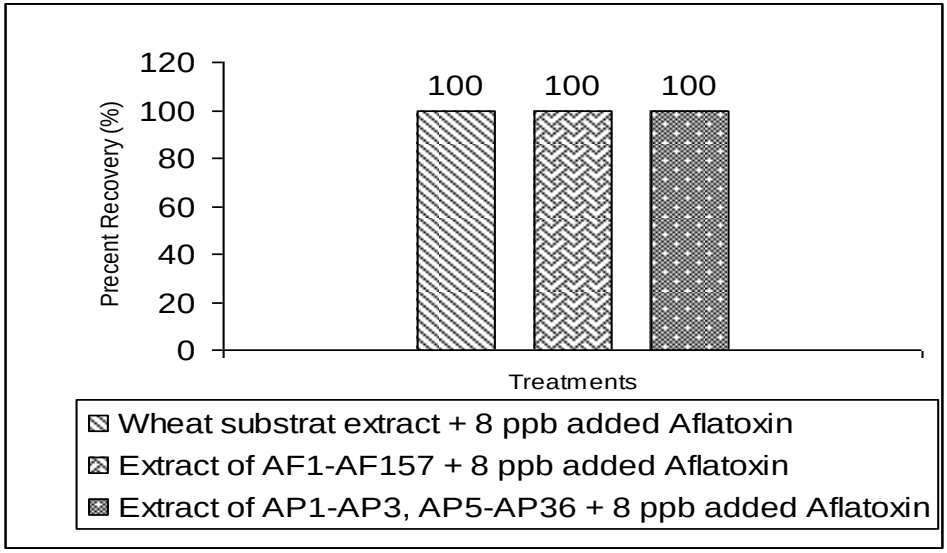


Fig. 10. Percent recovery of spiked control during *in vitro* study of Non Aflatoxinogenic *A. flavus* and *A. parasiticus* isolates.
Each bar value is the mean of three replicates of each treatment X values recorded for all analyzed batches. Error bars are the representatives of ±SE of each treatment.

Discussion

This survey revealed the absence of aflatoxigenic *A. flavus* isolates and only one AP4 out of 36 *A. parasiticus* and 157 *A. flavus* isolates was toxin producing strain. A survey of toxigenic fungi carried out by Bresler *et al*., (1995) demonstrated a low potential for Aflatoxin production by *A. flavus* since only 4 out of 34 strains were able to produce the toxin. Percent recovery of the toxin from spiked samples and spiked controls ranged from 98–100%. Joanna *et al*., (2001) observed 92% recovery of toxin by immunoaffinity chromatography with fluorescence detection. Presence of all the four genes in the Aflatoxinogenic AP4 isolate in the present study is in accordance with the findings of Criseo *et al*., (2001) but the absence of all the four tested genes at a time in all the nonaflatoxigenic strains of present study is contrary to their findings. Lee *et al*., (2006) suggested for safety reasons that aflR gene presence could be used to detect the Aflatoxin production by *A. flavus* strains. In the present study, some of the strains have aflR genes but they did not produce the Aflatoxin as compared to the findings of Kusumoto *et al*., (1998) who found PCR products of aflR homologs only when RNA of *A. parasiticus* was used as a template for amplification of aflR c DNA. At the same time when aflR homolog of *A. oryzae* strain was used no amplification was observed. Results of our studies are similar to the findings of Venden *et al*., (2001) who stated that all aflR and Omt 1 deleted strains of *A. flavus* turned out to be Aflatoxin non-producers. *A. flavus* did not show any strain that contains all the four bands for genes AflR, Omt 1, Ver1 and nor 1. AflR is a regulatory gene which was named as apa 1 gene in *A. parasiticus* strain (Yu *et al*., 1995 & 2004), while all the other three genes of this study are structural genes in the Aflatoxin biosynthetic pathway. The above mentioned discussion indicates that the conventional methods, although laborious and time consuming, are more reliable. Our ELIZA and PCR results compliment each other. Zachova (2003) also observed that only toxin producing strains had all the genes. On the contrary Yang *et al*., (2004) found different results by PCR and ELIZA, Aflatoxin detection was negative in all the samples while some of the tested samples by them have all the four genes nor a, ver 1, omt A anf avf A (AflR) genes.

Aflatoxin was mostly produced at 12% and 14% moistures and 25°C and 30°C in all the three trials of storage experiment for toxinogenic isolated strains of *A. parasiticus* strains and positive controls. Dawar & Ghaffar (1992) also designed experiments to study the effect of moisture and temperature on the development of mycoflora and subsequent Aflatoxin production. They found maximum *A. flavus* infection at 15% moisture content and maximum Aflatoxin concentration was detected at 30°C after 60 days of storage under controlled conditions. Lin *et al*., (1980) also studied the temperature and duration of incubation effects on the toxin production in sterilized corn grains. They concluded that maximum concentration was found in longer time stored grains and at the temperature of 25°C. Bilgrami *et al*., (1988) reported loss of toxigenicity of *Aspergillus flavus* strains during sub culturing. This phenomenon was observed in the aflatoxinogenic *A. parasiticus* strain in the present study. Although *A. flavus* and *A. parasiticus* both contain the Aflatoxin gene cluster with genes in the same sequential order (Ehrlich *et al.*, 2005; Yu *et al.*, 2004), data from different geographical areas demonstrate a great variability in the mycotoxin-producing potential of *A. flavus* and closely related species. According to Horn & Dorner (1999), knowledge of regional differences in the toxigenicity of *A. flavus* populations as well as knowledge of the association of these populations with the dominant crop in a region may be important in determining which control measures are most effective in reducing preharvest Aflatoxin contamination.

They reported that *A. flavus* was predominant than proportion of *A. parasiticus* similarly in the present study more *A. flavus* strains were isolated than isolates of *A. parasiticus.* Other authors have reported that nontoxigenic isolates of *A. parasiticus* are extremely rare similarly in this study among all the isolates only one *A. parasiticus* strain was found to be toxinogenic (Horn *et al.*, 1996; Tran-Dinh *et al*., 1999).

It is concluded from the present studies that *A. flavus* and *A. parasiticus* strains population invading wheat grains were all non aflatoxigenic ones except one *A. parasiicus* AP4 strain which was toxinogenic out of 157 *A. flavus* and 36 *A. flavus* isolates. In the present study atoxinogenic *A. flavus* and *A. parasiticus* isolates reduced the Aflatoxin production of Aflatoxinogenic *A. flavus* and *A. parasiticus* isolates during *in vitro* study, this shows that native non-aflatoxigenic strains could be organisms of interest in order to develop biocontrol strategies for reducing Aflatoxin contamination in other highly Aflatoxin contaminated crops after their thorough survey for assessment of their fungal and Aflatoxin contamination status. Its also stated that highly competitive atoxigenic strains might be applied to agricultural fields as biocompetitive agents (Egel *et al.*, 1994, Cotty & Cardwell, 1999; Horn & Dorner, 1999). Peter & Deepak (1994) studied the ability of atoxigenic *A. flavu*s strain to reduce the Aflatoxin contamination in the contaminated cotton bolls.

Acknowledgements

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