1 **Characterization of non-aflatoxigenic strains of *Aspergillus flavus* as potential**
2 **biocontrol agent for the management of aflatoxin contamination in groundnut**

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Abstract

In the present study, nine non-aflatoxigenic *Aspergillus flavus* strains were evaluated for their potential to reduce *A. flavus* infection and aflatoxin contamination in groundnut. Genetic characterization of these strains revealed six different deletion patterns (A-F) for thirteen examined genes from the aflatoxin biosynthesis pathway. Strain AFGS5 recorded maximum number of gene deletion (F) which included 12 out of 13 tested genes. Our findings indicated that aflR was the most frequently absent gene among the observed deletion patterns. A dendrogram inferred from combining random amplified polymorphic DNA and microsatellite data showed three of the non-aflatoxigenic strains segregating from other sampled isolates (aflatoxigenic and non-aflatoxigenic) tested. Greenhouse experiments, involving non-aflatoxigenic strains as biocontrol agents resulted in two strains, AFGS5 and AFGS12, which significantly reduced the population of aflatoxigenic fungi and the level of total aflatoxins in the rhizosphere/geocarposphere of soil samples as well as in groundnut seeds. Based on our findings, the use of these native non-aflatoxigenic strains; AFGS5 or AFGS12 in particular, as biopesticides could offer efficacious mitigation of aflatoxin contamination.

Key words: PCR, Deletion pattern, Rhizosphere, biopesticides.

Introduction

Aflatoxins (AFs) are a group of low molecular weight, polyketide-derived secondary metabolites primarily produced by *Aspergillus flavus*, *A. parasiticus* and *A. nomius* [1, 2]. Chemically these toxins are dihydrofuranofuran and tetrahydrofuran moieties fused with a substituted coumarin. Among the AFs, AFB₁ is the most potent toxin, that shows hepatotoxic, teratogenic and mutagenic properties causing hepatitis, haemorrhage, oedema, immunosuppression and hepatic carcinoma in mammals [3, 4].

Infection and subsequent aflatoxin contamination of agricultural commodities by these fungi pose major economical and health concerns for growers and consumers, respectively. Several developed countries enforce strict regulation on the import of agricultural commodities with aflatoxin contamination. This development negatively impacts the exportation of commodities from under-developed and developing countries whose regulation of aflatoxin contamination is not as strictly monitored. Such contamination can be detrimental to the export of these commodities and the economy of the country. Among the crops susceptible to AF contamination, groundnut (*Arachis hypogaea* L.) susceptibility is linked to unique seed production process and its chemical composition which facilitate fungal proliferation and toxin production [5] in both pre- and post-harvest conditions [6]. Also, the environment and practices of groundnut growing regions of India such as hot and humid growing conditions, unexpected droughts, pre-harvest fungal infection, poor storage conditions and lack of awareness among farmers heightens the risks of aflatoxin contamination [7]. Hence, management of aflatoxigenic fungi and AF contamination in groundnut is of prime importance.

Chemical fungicides and fumigants are being frequently applied during pre- and post-harvest conditions, to manage AF contamination in groundnut. However, the risk associated with

excess chemical use on human and animal health as well as the environment [8], has forced producers and consumers to turn to safer ways of AF management. Attempts have been made to develop eco-friendly management strategies such as improved cultural practices, processing and storage conditions, breeding for host resistance and the use of antagonistic microorganisms [9, 10]. One of the recent approaches which has proved effective under field conditions is the use of non-aflatoxigenic fungal strains which competitively exclude aflatoxigenic fungi [11, 12]. Afla-Guard® (*A. flavus* strain NRRL 21882) is the first commercialized non-aflatoxigenic strain followed by AF36 (*A. flavus* strain NRRL 18543) and K49 (*A. flavus* strain NRRL 30797) recommended for use in groundnut, maize and cotton production, respectively, in USA [13,14]. Similarly, in different parts of Africa, Aflasafe™, which is a mixture of four native atoxigenic strains, is in use to manage AF in maize and groundnut production [15]. Further, bioplastic based granular formulation of non-aflatoxigenic *A. flavus* was developed and successfully tested under field conditions as soil and foliar applications [16,17]. In India, research regarding isolation and characterization of non-aflatoxigenic fungi and its use in managing AF and aflatoxigenic fungi in agricultural crops is generally ignored. Locally adapted non-aflatoxigenic strains are thought to be necessary for optimal efficacy and long-term modifications to fungal community structures. Hence, the present study was aimed to: (i) genetically characterize native non-aflatoxigenic fungal strains isolated from seeds and rhizosphere/geocarposphere soil of groundnut and (ii) evaluate the potential of these isolates to competitively exclude the toxigenic strains and reduce AF contamination in groundnut.

Materials and methods

Fungal strains

Here we used twenty one *A. flavus* strains among which, seventeen strains were originally isolated from groundnut seeds collected from different agroclimatic regions of India [18, Table 1], Strains AFGS5 and AFGS12 were isolated from rhizosphere/geocarposphere soil of groundnut plants and two reference atoxigenic strains of *A. flavus*, NRRL32353 and NRRL32354, were kindly gifted by Jim Swezey, Collection Manager, USDA, ARS, Peoria, IL. *Aspergillus flavus* isolates were identified by observing the morphological characters under a stereo binocular microscope, conidia and conidiophore characters under compound microscope and by analyzing the sequence of ITS regions amplified using primers ITS1 and ITS4 [19]. Throughout the experimental period all the *A. flavus* isolates were maintained on Potato dextrose agar (PDA) and sub-cultured every 60 days. For all laboratory and greenhouse experiments, aflatoxigenic *A. flavus* strain AFG1 was used as a positive control.

Aflatoxigenicity of *Aspergillus flavus* strains

Ultraviolet and ammonia vapor tests were performed as per the procedures of Hara et al. [20], and Saito and Machida [21], respectively. An agar plug from 7-day old fungal culture was extracted with chloroform and AF was detected by separating the metabolites on a pre-coated thin layer chromatographic plate [22]. For indirect competitive (ic) ELISA, *A. flavus* isolates were grown on potato dextrose broth (PDB; 50 ml) in 100 ml conical flasks for 7 days at 28 ± 2 °C. At the end of incubation period, different dilutions (1:1, 1:10 and 1:100) of culture filtrate in methanol:water (7:3 v/v) were analyzed for the presence of AF as below.

Stability of non-aflatoxigenicity in *Aspergillus flavus* strains

Three single-spore colonies from each of the nine non-aflatoxigenic isolates were established on PDA. A single spore from each culture was transferred on to PDA medium, cultured for 1 week (28 ± 2 °C) and again transferred by single spore. Similar serial transfers were repeated 20 times for each isolate. Cultures starting with the fifth generation, and each fifth generation through the 20th, were tested by icELISA for AF production.

DNA extraction

Each isolate was grown on PDB for 4-5 days on a rotary shaker at 150 rpm then mycelium was harvested by washing with sterile water. DNA extraction was carried out following the method of Saitoh et al. [23] with minor modifications. Briefly, one g of mycelia was powdered with pre-chilled pestle and mortar using liquid nitrogen, 500 µl of lysis buffer (200 mM l⁻¹ of Tris-HCl, 50 mM l⁻¹ of EDTA, 200 mM l⁻¹ of NaCl, 1% (w/v) of SDS, pH 8.0) was added and incubated for 1 h at 65 °C. After centrifuging at 10,000 rpm for 10 min at 4 °C, 800 µL supernatant were transferred into a fresh microtube and mixed with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v). The supernatant was transferred into fresh tube and one volume of cold isopropanol was added to precipitate the DNA and kept at -20 °C overnight then centrifuged at 10,000 rpm for 5 min at 4 °C. After one wash with 70% ethanol, the DNA pellet was air-dried and dissolved in 25 µl of 100 mM l⁻¹ of Tris-HCl buffer, and DNA concentration was measured with a NanoDrop (Thermo Scientific).

Genetic analysis of deletion patterns in the aflatoxin biosynthesis gene cluster

Polymerase chain reaction (PCR) was performed according to Jiang et al. [24] with 13 sets of primers specific for AF biosynthesis genes (Table 2). The typical PCR 25 µl mixture containing:

157 20 ng of DNA template, 0.2 μ M each primer, 0.5 μ l of 10 mM dNTP mix (10 mM each of dATP,
158 dCTP, dGTP, dTTP; Bangalore Genei), 2 μ l of reaction buffer (20 mM Tris-HCl (pH 8.8), 10
159 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgSO_4 , 0.1% Triton X-100) and 0.5 U of *Taq* DNA
160 polymerase. A total of 30 PCR cycles with the following temperature regimen was performed:
161 initial pre-heating for 2 min at 95 °C, followed by denaturation at 95 °C for 30 sec, annealing at
162 55 °C for 40 sec and extension at 72 °C for 90 sec and termination with a final extension at 72 °C
163 for 5 min. The PCR products were separated on 1.5% agarose gel in Tris-borate EDTA (TBE)
164 buffer and documented after staining with ethidium bromide. Presence or absence of PCR
165 products was recorded for each primer pair.

166 Random-amplified polymorphic DNA (RAPD) and microsatellite-primed PCR (MP-PCR)
167 analysis

168 Genetic diversity among 19 isolates of *A. flavus* was assessed by RAPD technique with 25
169 oligonucleotides from the RFu-D primer series (Bangalore Genei) (Table 3) and MP-PCR
170 analysis was carried out with 10 primer sets (Table 4). PCR amplifications were performed in a
171 total volume of 25 μ l of reaction mixture containing 25 ng of DNA template, 20 pmol primer, 0.5
172 μ l of 10 mM dNTP mix (10 mM each of dATP, dCTP, dGTP, dTTP; Bangalore Genei), 2 μ l of
173 reaction buffer (20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgSO_4 ,
174 0.1% Triton X-100) and 0.5 U of *Taq* DNA polymerase. The following PCR parameters were
175 used for RAPD: Initial denaturation at 94 °C for 3 min followed by 45 cycles at 94 °C for 1 min,
176 35 °C for 1 min and 72 °C for 2 min and a final extension step at 72 °C for 10 min. For MP-
177 PCR, annealing temperatures were adjusted depending on the G + C contents and program was:
178 initial DNA denaturation at 94 °C for 2 min followed by 40 cycles of DNA denaturation for 1
179 min at 94 °C, 1 min at annealing temperature specific for each primer and extension for 1.5 min

at 72 °C, with final extension for 6 min at 72 °C. The PCR products were separated on 1.5% agarose gels in Tris-borate EDTA (TBE) buffer and documented after staining with ethidium bromide.

Bands of RAPD and MP-PCR products were scored as binary digit “1” and “0” which stand for presence and absence of amplified product. Cluster analysis was performed using Tools for population genetic analyses (TFPGA) version 1.3. The generated pair wise similarity matrix was used to group strains by the unweighted pair-group method with arithmetic average (UPGMA). A dendrogram was derived from the similarity matrix.

Greenhouse study

For greenhouse studies the fungal inoculum was prepared by growing on PDA plates for 7-9 days at $28 \pm 2^\circ\text{C}$. An aliquot of 10 ml of sterile distilled water was added to each plate and conidia were gently freed from the culture surface by shaking. The remaining conidia were dislodged with a sterile brush and the suspension was collected in a 100 ml conical flask. The conidial concentration was adjusted to 1×10^8 conidia ml^{-1} using a haemocytometer.

Greenhouse experiments were conducted with natural light and temperatures of $18 \pm 2^\circ\text{C}$ during night and $28 \pm 2^\circ\text{C}$ in day. The potting mixture, consisting of soil:sand:farm yard manure (2:1:1 v/v/v), was sterilized by autoclaving at 121°C (15 psi) for 45 min and cooled. The potting mixture was amended with conidia of isolate AFG1 (aflatoxigenic) and conidia of each of non-aflatoxigenic isolates (at 2.5×10^5 conidia g^{-1} soil of each isolate for a total of 5×10^5 conidia g^{-1} soil). Pots of 20 cm diameter were filled with potting mixture to three-fourths capacity (2 kg). A positive control containing only aflatoxigenic strain AFG1 (5×10^5 conidia g^{-1} soil) and a negative control without *A. flavus* was also maintained. Groundnut seedlings were raised from *A.*

202 *flavus* free seeds (cv.TMV-2) at the rate of one seedling per pot. At the end of experimental
203 period (4 months after sowing), seedlings were uprooted without damaging the roots and pods
204 and rhizospheric/geocarpospheric soil samples were collected. Pods were sun dried and stored in
205 cotton bags for 3 months at room temperature. In the greenhouse experiments, for each treatment
206 there were three replicates incorporating eight pots. Soil and seed from each replicate was
207 analyzed for aflatoxin and aflatoxigenic fungi, separately. The whole greenhouse experiment was
208 repeated thrice with the controls.

209 Towards the end of experimental period groundnut plants were uprooted without
210 damaging the root system along with adhered soils. Rhizospheric/geocarpospheric soil samples
211 from all treatments were collected. The soil samples were serially diluted and different dilutions
212 were spread plated on PDA medium amended with streptomycin (300 mg/l). The plates were
213 incubated for five days at $28\pm 2^{\circ}\text{C}$ under dark conditions. Each colony from different dilutions
214 was pure cultured and analyzed for AF production by icELISA as explained above. The total
215 number of aflatoxigenic and non-aflatoxigenic strains present in one g of soil was calculated for
216 each treatment.

217 Similarly, aflatoxin from rhizospheric/geocarpospheric soil samples was extracted
218 following the procedure of Kumar et al. [25]. Briefly, 25 g samples were placed in a sealed flask
219 to which 100 ml of chloroform:distilled water (3:1 v/v) were added. The samples were incubated
220 in a shaker at 100 rpm at room temperature for 12 h. The samples were then filtered through
221 Whatman No. 1 filter paper to collect chloroform layer and filtrates were concentrated using a
222 rotary evaporator. The collected chloroform extract was concentrated using a rotary evaporator
223 and the concentration of AF was quantified by icELISA.

After a storage period of three months, aflatoxin was quantified in seeds collected from different treatments. Seed-borne incidence of *A. flavus* was determined following a standard blotter method [26]. To determine the toxigenicity of fungi growing on each seed, sub isolates were taken from four randomly selected points on each seed and pure cultured onto PDA. Aflatoxigenicity of each isolated fungi was determined through icELISA.

Indirect competitive enzyme linked immunosorbent assay (icELISA)

Total AF content was estimated from culture filtrate, soils and groundnut seeds by modified method of Reddy et al. [27], and Hariprasad et al. [28]. AFB₁-BSA conjugate was prepared following the standard procedure [29]. To perform icELISA, the wells of microtitre plates (Maxi-sorp F96, NalgeNunc International, Roskilde, Denmark) were coated overnight at 4 °C with 100 µl of AFB₁-BSA conjugate in carbonate buffer (1 mg ml⁻¹) at pH 9.6, and then washed thrice with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST). Plates were blocked with 5% ovalbumin (OVA) in carbonate buffer (pH 9.6). AFB₁ standard (Sigma) in 10% (v/v) methanol-PBS (50 µl) or different dilutions of samples (50 µl) was added to the wells. After 15 min incubation at 37 °C, 100 µl of anti-AFB₁ antibody (Sigma) (1:10000) in PBS were added and incubated at 37 °C for 45 min. The plates were washed with PBST 4-5 times. Subsequently, 100 µl of secondary antibody conjugated with horseradish peroxidase (HRP) (1:10000) (Bangalore Genei) in PBS were added and incubated at 37 °C for 45 min. At the end of the incubation period the plates were washed 4-5 times with PBST. One hundred µL of substrate (TMB-H₂O₂) (Bangalore Genei) was added and incubated at room temperature for 10-15 min. The reaction was stopped by adding 100 µl of stop solution (2 M H₂SO₄) and the color developed was read at 450 nm. Standard curves using absorbance (A) vs. logarithm of analyte

concentration were plotted. AF contents in the samples were determined from the standard curve and expressed in ppb.

Statistical analysis

The data were subjected to arcsine transformation and analysis of variance using SPSS, version 16. The significant differences between the treatment means were compared using Highest Significant Difference (HSD) as obtained by Tukey's test at $P \leq 0.05$ level.

Results

Aflatoxigenicity of *Aspergillus flavus* strains

The UV, ammonia vapor test, TLC and icELISA were generally consistent in differentiating aflatoxigenic and non-aflatoxigenic strains of *A. flavus* as represented in Table1. Results obtained from icELISA confirmed that, all nine non-aflatoxigenic *A. flavus* strains did not produce AF and their non-aflatoxigenicity was confirmed up to 20 subcultures.

Deletion pattern of aflatoxin biosynthesis gene cluster

Upon PCR amplification of 13 target genes, it was confirmed that aflatoxigenic strain AFG1 contained all of the tested genes. Nine non-aflatoxigenic strains exhibited six different deletion patterns (Table 1; Fig. 1). Except AFG15, all non-aflatoxigenic strains showed a common deletion in gene *aflR*. In contrast, non-aflatoxigenic strain AFGS5 only amplified for the *glcA* gene. Primer pair for *norB-cypA* generated amplification product with only two non-aflatoxigenic strains, AFG26 and AFG64. Largest deletion of AF biosynthesis cluster was

recorded in strain AFGS5 which includes 12 out of 13 tested genes (Table 2), whereas in strain AFG15 deletion was observed only in norB-cypA region.

Random-amplified polymorphic DNA (RAPD) and microsatellite-primed PCR (MP-PCR) analysis

The 25 tested RAPD primers generated 287 bands which ranged from 250 bp to 900 bp. The number of amplified bands per primer ranged between 4 and 21. Percent polymorphism of RAPD primers ranged from 61 to 100, with eight primers (Rfu-4, Rfu-6, Rfu-7, Rfu-11, Rfu-13, Rfu-14, Rfu-18, and Rfu-20) displaying 100% polymorphism (Table 3). Ten MP-PCR primers generated 120 bands, and the number of fragments produced per primer ranged from 7 to 16. A maximum number of bands (16) were produced by primer UBC812, of which eight were polymorphic. The percent polymorphism exhibited by MP-PCR primers ranged from 50 to 100% (Table 4).

Combined dendrogram analysis resulted in three major groups with genetic similarity 44.7-92.1% (Fig. 2). The first group included 13 isolates and highest similarity (92.1%) was exhibited by isolates AFG64(-) and AFG62(+). The second group included three strains, AFGS15(+), AFGS5(-) and (-), AFG71 which included both aflatoxigenic and non-aflatoxigenic isolates. The third group included three isolates, AFG57(+), AFGS12(-) and AFG8(+) as outliers which showed similarity range of 61.4-69.8%.

Greenhouse study

Analysis of rhizospheric/geocarpospheric soil samples collected from different co-inoculation treatments revealed the presence of aflatoxigenic fungi and variation in AF concentrations. Non-aflatoxigenic fungal strains AFGS5 and AFGS12 significantly ($P \leq 0.05$) reduced the presence of the competing aflatoxigenic strain (3.30 and 3.99 Log cfu g⁻¹ soil respectively) and also AF

levels reduced to 4 and 5 ppb respectively in the rhizosphere/geocarposphere in comparison with other treatments and standard non-aflatoxigenic strains (NRRL32353 and NRRL32354) (Table 5). Similar results were also observed in both harvested and stored seeds, where AFGS5 and AFGS12 significantly ($P \leq 0.05$) reduced AF levels to 2.0 and 0.0 ppb, respectively when compared to the positive control and standard atoxigenic fungi. There was however no significant ($P \leq 0.05$) difference in seed borne incidence of *A. flavus* between the treatments. Additionally, seed-borne isolates from treatments with AFGS5 and AFGS12 were predominantly non-aflatoxigenic (Table 5).

Discussion

One major advancement in pre- and post-harvest management of AF and aflatoxigenic fungal contamination is the use of non-aflatoxigenic strains of *A. flavus* or *A. parasiticus* as biocompetitors of aflatoxigenic fungi [30, 31]. In nature afltoxigenic and non-aflatoxigenic fungal strains are having equal opportunity to survive and multiply in given medium. But non-aflatoxigenic strains have slightly upper hand because they don't spend energy in synthesizing aflatoxin [32]. Non-aflatoxigenic fungal strains occupy the same niche and compete for space and nutrition with aflatoxigenic strains thereby reduce the population of later and subsequent aflatoxin level in infected grains/seeds. The phenomenon of competitive exclusion is primarily depends on adaptability to local conditions and aggressive growth of non-aflatoxigenic strains [33]. Extensive research on isolation and characterization of non-aflatoxigenic fungi have resulted in only a limited number of isolates for commercial applications.

The advantage of AF production by these fungi in improving their ecological fitness is yet to be explained. Aflatoxin biosynthesis in these fungi is affected by external environmental and nutritional factors including the developmental status [34, 35]. It was found that 25

contiguous genes in a 70-kb cluster are situated at the telomeric region on chromosome III which are involved in different steps of AF biosynthesis [36]. Under variable environmental conditions, non-aflatoxigenic strains are spontaneously generated due to recombination, inversions, deletions, translocations and other genomic rearrangements [37]. Natural occurrence of non-aflatoxigenic *A.flavus* isolates with partial, or entire deletion of genes from the AF biosynthetic cluster is not rare and the resulting deletion patterns are diverse [38]. Recently, Jiang et al. [24] reported an 89.59 kb deletion in the AF biosynthesis gene cluster of *A. flavus* AF051, and further analysis revealed that the deleted region was replaced by 3.83 kb DNA, which was non-homologous to any of the genes present in a typical aflatoxigenic strain. A large deletion in the AF biosynthesis gene cluster and its replacement with non-homologous gene can serve as a safeguard in preventing genetic reversion or recombination, thereby ensuring its non-aflatoxigenicity under varied environmental conditions. In our studies, nine non-aflatoxigenic strains of *A. flavus* showed 6 patterns of deletion (A-F) in AF biosynthesis gene cluster. Among them B, E and F patterns had larger deletions in the AF biosynthesis gene cluster. Pattern F observed only with strain AFGS5, had largest deletion, from gene *C1* to *hypA*, or 12 out of 13 genes tested (Fig. 1).

Previous RAPD and ISSR techniques revealed higher levels of genetic diversity among aflatoxigenic and non-aflatoxigenic strains [39, 40], but attempts to develop a molecular marker that easily distinguish between these chemotypes failed. Yet, in the present study, we observed greater genetic diversity among aflatoxigenic and non-aflatoxigenic strains. There was no correlation between aflatoxigenicity and RAPD and MP-PCR pattern.

In our studies, nine non-aflatoxigenic strains of *A. flavus* were evaluated for their ability to suppress the growth and proliferation of aflatoxigenic strains under greenhouse conditions.

During greenhouse studies it was observed that two native strains (AFGS5 and AFGS12) significantly reduced rhizospheric population of aflatoxigenic strains and AF level and also recorded significant ($P \leq 0.05$) reduction in aflatoxigenic fungal infection and AF contamination in seeds. Recently, similar observations have been reported in an interaction study between a green fluorescent protein (GFP) tagged aflatoxigenic strain of *A. flavus* AF70 with non-aflatoxigenic *A. flavus* strain AF36 in co-inoculated maize kernels [33].

This study demonstrated the biocontrol potential of two native non-aflatoxigenic strains, AFGS5 and AFGS12 against aflatoxigenic *A. flavus* infection and AF contamination in groundnut. In our greenhouse studies, none of the treatments showed reduction in total *Aspergillus* seed infection. This may be because, non-aflatoxigenic fungal strains are equally or more efficient in infecting and colonizing in seeds without producing AF. But being seed-borne fungi the non-aflatoxigenic strains may deteriorate seed/grain quality [33]. By analyzing the results obtained from the present study it can be concluded that, use of non-aflatoxigenic strains AFGS5 and AFGS12 in field studies is more appropriate, as they have a large deletion in AF biosynthesis gene cluster and also show aggressive growth over aflatoxigenic strains in rhizosphere soil as well as on seeds. However as a precautionary measure need to these two isolates analyzed for the production of other toxigenic metabolites such as indole-diterpenes, aflatrem, paxillenes, paspalicines, aflavinines, cyclopiazonic acid and pseurotin before their field application.

Acknowledgments

355 This research was financially supported by the Department of Biotechnology (DBT),
356 Government of India (Project-BT/PR9864/AGR/05/391/2007; Dated: 6 June 2008), India. The
357 authors wish to thank the Chairman, Department of Biotechnology, University of Mysore,
358 Manasagangotri, Mysore 570006, India and Jim Swezey, Collection Manger, USDA, ARS,
359 Peoria, IL., for providing non-aflatoxigenic strains of *Aspergillus flavus* NRRL32353 and
360 NRRL32354.

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References

- 1) Smith, J.E., & Moss, M.O. (1985). *Mycotoxins: Formation, analysis, and significance*. John Wiley and Sons, New York, pp. 148.
- 2) Bayman, P., & Cotty P.J. (1993). Genetic diversity in *Aspergillus flavus*: association with aflatoxin production and morphology. *Can J Bot*, 71, 23-34.
- 3) Turner, P.C., Sylla, A., Gong, Y.Y., Diallo, M.S., Sutcliffe, A.E., Hall, A.J., & Wild, C.P. (2005). Reduction in exposure to carcinogenic aflatoxins by postharvest intervention measures in West Africa: a community-based intervention study. *Lancet*, 365, 1950-1956.
- 4) Lutfullah, G., & Hussain, A. (2012). Studies on contamination level of aflatoxins in some cereals and beans of Pakistan. *FoodControl*, 23, 32-36.
- 5) Savage, G.P., & Keenan, J.I. (1994). The composition and nutritive value of groundnut kernels, In: Smart, J. (ed). *The Groundnut crop: scientific basis for improvement*. London: Chapman and Hall, pp. 173-213.
- 6) Abbas, H.K., Zablotowicz, R.M., Horn, B.W., Phillips, N.A., Johnson, B.J., Jin, X., & Abel, C.A. (2011). Comparison of major biocontrol strains of non-aflatoxigenic *Aspergillus flavus* for the reduction of aflatoxins and cyclopiazonic acid in maize. *Food Addit Contam Part A. Chem Anal Control Expo Risk Assess*, 28, 198-208.
- 7) Reddy, B.N., & Raghavender, C.R. (2007). Outbreaks of aflatoxicoses in India. *Afr J Food Agric Nutr Dev*, 7, 15-27.
- 8) Cleveland, T.E., Dowd, P.F., Desjardins, A.E., Bhatnagar, D., & Cotty, P.J. (2003). United States Department of Agriculture-Agricultural Research Service research on pre-harvest

- prevention of mycotoxins and mycotoxigenic fungi in US crops. Pest Manag Sci, 59, 629-642.
- 9) Korsten, L. (2006). Advances in control of postharvest diseases in tropical fresh produce. International Journal Postharvest TechnolInnov, 1, 48-61
- 10) Velmourougane, K., Bhat, R., Gopinandhan, T.N., & Panneerselvam, P. (2011). Management of *Aspergillus ochraceus* and Ochratoxin-A contamination in coffee during on-farm processing through commercial yeast inoculation. Biol Control, 57, 215-221.
- 11) Dorner, J.W., Cole, R.J., Connick, W.J., Daigle, D.J., Mcguire, M.R., & Shasha, B.S. (2003). Evaluation of biological control formulations to reduce aflatoxin contamination in peanuts. Biol Control, 26, 318-324.
- 12) Zanon, A., Chiotta, M., Giaj-Merlera, G., & Barros, G. (2013). Evaluation of potential biocontrol agent for aflatoxin in Argentinean peanuts. Int J Food Microbiol, 162, 220-225.
- 13) Accinelli, C., & Abbas, H.K. (2011). New perspectives for the application of bioplastic materials in the biocontrol of *Aspergillus flavus* in corn. Toxin Rev, 30, 71-78.
- 14) Abbas, H.K., Wilkinson, J.R., Zablotowicz, R.M., Accinelli, C., Abel, C.A., Bruns, H.A., & Weaver, M.A. (2009). Ecology of *Aspergillus flavus*, regulation of aflatoxin production and management strategies to reduce aflatoxin contamination of corn. Toxin Rev, 28, 142-153.
- 15) Probst, C., Bandyopadhyay, R., Price, L.E., & Cotty, P.J. (2011). Identification of atoxigenic *Aspergillus flavus* isolates to reduce aflatoxin contamination of maize in Kenya. Plant Disease, 95, 212-218.

- 407 16) Accinelli, C., Saccà, M.L., Abbas, H.K., Zablotowicz, R.M., & Wilkinson, J.R. (2009). Use
408 of a granular bioplastic formulation for carrying conidia of a non-aflatoxigenic strain of
409 *Aspergillus flavus*. Bioresources Technol, 100, 3997-4004.
- 410 17) Accinelli, C., Abbas, H.K., Vicari, A., & Shier, W.T., (2016). Leaf application of a sprayable
411 bioplastic-based formulation of biocontrol *Aspergillus flavus* strains for reduction of
412 aflatoxins in corn. Pest Manag Sci, 72, 1521–1528.
- 413 18) Navya, H.M., Hariprasad, P., Naveen, J., Chandranayaka, S., & Niranjana, S.R. (2013).
414 Natural occurrence of aflatoxin, aflatoxigenic and non-aflatoxigenic *Aspergillus flavus* in
415 groundnut seeds across India. Afr J Biotechnol, 12, 2587-2597.
- 416 19) White, T.J. Bruns, T., Lee, S., & Taylor, J.W. (1990). Amplification and direct sequencing of
417 fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods
418 and Applications, eds. Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Academic
419 Press, Inc., New York, pp. 315-322.
- 420 20) Hara, S., Fennel, D.I., & Hesseltine, C.W. (1974). Aflatoxin-producing strains of *Aspergillus*
421 *flavus* detected by fluorescence of agar medium under ultraviolet light. Appl Microbiol,
422 27, 1118-1123.
- 423 21) Saito, M., & Machida, S. (1999). A rapid identification method for aflatoxin-producing
424 strains of *A. flavus* and *A. parasiticus* by ammonia vapor. Mycoscience, 40, 205-211.
- 425 22) Criseo, G., Bagnara, A., & Bisignano, G. (2001). Differentiation of aflatoxin producing and
426 nonproducing strains of *Aspergillus flavus* group. Lett Appl Microbiol, 33, 291-295.
- 427 23) Saitoh, K., Togashi, K., Arie, T., & Teraoka, T. (2006). A simple method for a mini-
428 preparation of fungal DNA. J Gen Plant Pathol, 72, 348-350.

- 429 24) Jiang, H., Yan, L., & Ma, Z. (2009). Molecular characterization of an atoxigenic *Aspergillus*
430 *flavus* strain AF051. Appl Microbiol Biotechnol, 107, 1857-1865.
- 431 25) Kumar, S., Shekar, M., Khan, A.A., & Sharma, P. (2007). A rapid technique for detection of
432 toxigenic and non-toxigenic strains of *Aspergillus flavus* from maize grains. Indian
433 Phytopath, 60, 31-34.
- 434 26) International seed testing association (ISTA) (2005). Proceedings of the International seed
435 testing association. International rules of seed testing. Seed Sci Technol, 15A, 1-9.
- 436 27) Reddy, K.R.N., Reddy, C.S., & Muralidharan, K. (2009). Detection of *Aspergillus* spp. and
437 aflatoxin B₁ in rice in India. Food Microbiol, 26, 27-37.
- 438 28) Hariprasad, P., Durivadivel, P., Snigdha, M., & Venkateswaran, G. (2013). Natural
439 occurrence of aflatoxin in green leafy vegetables. Food Chem, 138, 1908-1913.
- 440 29) Kolosova, A.Y., Shim, W.B., Yang, Z.Y., Eremin, S.A., & Chung, D.H. (2006). Direct
441 competitive ELISA based on a monoclonal antibody for detection of aflatoxin B₁.
442 Stabilization of ELISA kit component and application to grain sample. Anal Bioanal
443 Chem, 384, 286-294.
- 444 30) Antilla, L., & Cotty, P.J. (2002). The ARS-ACRPC partnership to control aflatoxin in
445 Arizona cotton: current status. Mycopathologia, 155, 64.
- 446 31) Dorner, J.W. (2004). Biological control of aflatoxin contamination of crops. J Toxicol Toxin
447 Rev, 23, 425-450.
- 448 32) Ehrlich, K., Wei, Q., Brown, R.L., & Bhatnagar, D. (2011). Inverse correlation of ability to
449 produce aflatoxin and *Aspergillus* colonization of maize seed. Food Nutr. Sci, 2, 486-
450 489.

- 33) Hruska, Z., Rajasekaran, K., Yao, H., Kincaid, R., Darlington, D., Brown, R.L., & Cleveland, T.E. (2014). Co-inoculation of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavus* to study fungal invasion, colonization and competition in maize kernels. *Front Microbiol*, 5, 1-5.
- 34) Guzman-de-Pena, D., Aguirre, J., & Ruiz-Herrera, J. (1998). Correlation between the regulation of sterigmatocystin biosynthesis and sexual sporulation in *Emericella nidulans*. *Antonie van Leeuwenhoek*, 73, 199-205.
- 35) Cary, J.W., Linz, J.E., & Bhatnagar, D. (2000). Aflatoxin: biological significance and regulation of toxin synthesis. In: Cary, J., Bhatnagar, D., Linz, J.E., eds. *Microbial food borne diseases; Mechanisms of pathogenesis and toxin synthesis*. Lancaster, Pennsylvania: Technomic Publishing, pp. 317-362
- 36) Yu, J., Chang, P.K., Ehrlich, K.C., Cary, J.W., Bhatnagar, D., Cleveland, T.E., Payne, G.A., Linz, J.E., Woloshuk, C.P., & Bennett, J.W. (2004). Clustered pathway genes in aflatoxin biosynthesis. *Appl Environ Microbiol*, 70, 1253-1262.
- 37) Carbone, I., Ramirez-Prado, J.H., Jakobek, J.L., & Horn, B.W. (2007). Gene duplication, modularity and adaptation in the evolution of the aflatoxin gene cluster. *BMC Evolutionary Biology*, 7, 111.
- 38) Chang, P.K., Horn, B.W., & Dorner, J.W. (2005). Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates. *Fungal Genet Biol*, 42, 914-923.
- 39) Lourenco, A., Durigon, E.L., Zanotto, P., Madeira, J.E.G.C., De Almeida, A.P., & Correa, B. (2007). Genetic diversity of environmental *Aspergillus flavus* strains in the state of Sao

473 Paulo, Brazil by random amplified polymorphic DNA. MemInst Oswaldo Cru,102, 687-
474 692.

475 40) Hatti, A.D., Taware, S.D., Taware, A.S., Pangrekar, P.P., Chavan, A.M., & Mukadam, D.S.
476 (2010). Genetic diversity of toxigenic and non-toxigenic *Aspergillus flavus* strains using
477 ISSR markers. International Journal of Current Research, 5, 061-066.

478

479 **Table 1** *Aspergillus flavus* strains used and their aflatoxigenicity
480

Isolate Code	Conventional methods		TLC	ELISA	Deletion Pattern	Aflatoxin production	Accession No.
	UV test	AV test					
AFG1	+	+	B ₁ , B ₂	+	-	<i>Positive</i>	KC491415
AFG8	+	+	B ₁	+	-	<i>Positive</i>	NS
AFG15	-	-	-	-	A	<i>Negative</i>	KC491416
AFG16	+	+	B ₁	+	-	<i>Positive</i>	NS
AFG19	+	+	-	+	-	<i>Positive</i>	NS
AFG20	-	-	-	-	B	<i>Negative</i>	KC491417
AFG26	-	-	-	-	C	<i>Negative</i>	KC491418
AFG39	+	+	-	+	-	<i>Positive</i>	NS
AFG48	-	-	-	-	B	<i>Negative</i>	KC491419
AFG49	+	+	B ₁	+	-	<i>Positive</i>	NS
AFG57	V	V	B ₁ , B ₂	+	-	<i>Positive</i>	NS
AFG62	+	V	B ₁ , B ₂	+	-	<i>Positive</i>	NS
AFG64	-	-	-	-	D	<i>Negative</i>	KC491420
AFG65	-	-	-	-	E	<i>Negative</i>	KC491421
AFG69	+	+	B ₁ , B ₂	+	-	<i>Positive</i>	NS
AFG71	-	-	-	-	B	<i>Negative</i>	KC491422
AFGS15	+	+	B ₁ , B ₂	+	-	<i>Positive</i>	NS
AFGS5	-	-	-	-	F	<i>Negative</i>	KC491423
AFGS12	-	-	-	-	E	<i>Negative</i>	KC491424

481 V: Variable, UV: Ultraviolet test, AV: Ammonia vapor test, TLC: Thin layer chromatography,
482 ELISA: Enzyme linked immune sorbent assay, AF: *Aspergillus flavus*, NS: Not submitted.

483 **Table 2** A list of specific primers used for the amplification of aflatoxin biosynthesis genes

Primer	Sequence	Size of PCR product (bp)	Relevant characters	Reference
C1-F	CCGTTGCAGTTAAACTCACA	409	The primer pair for amplification of partial aflatoxin gene <i>C1</i>	[22]
C1-R	GAAGCTCGATCTTCTCCATGA			
C3-F	TCTGGAGTCGGAGGTTAGGTT	544	The primer pair for amplification of partial aflatoxin gene <i>C3</i>	[22]
C3-R	GAGCAACACGATCATTGCAT			
norB-cypA-F	GTGCCCAGCATCTTGGTCCA	800	The primer pair for amplification of partial aflatoxin gene <i>nor B</i> and <i>cypA</i>	[39]
norB-cypA-R	AGGACTTGATGATTTCCTCGTC			
aflT-F	TGCGGACATCTAACGACCAT	750	The primer pair for amplification of partial aflatoxin gene <i>afl T</i>	[22]
aflT-R	AGGTCACTTCGTTTCGTGAAGG			
pksA-F	GCTGGGATTCTGCATGGGTT	399	The primer pair for amplification of partial aflatoxin gene <i>pks A</i>	[40]
pksA-R	CAGTTGCTCCCAAGGAGTGGT			
aflR-F	TGAGAAAGGGGACGCTGGAT	873	The primer pair for amplification of partial aflatoxin gene <i>afl R</i>	[22]
aflR-R	CAATCGAATCAACCACCACA			
norA-F	CCTTATGCCTGGGAACGAT	399	The primer pair for amplification of partial aflatoxin gene <i>nor A</i>	[40]
norA-R	TTCGCATCACTTCCTCCACA			
Ver1-F	ACCACCGTTTAGATGGCAAA	472	The primer pair for amplification of partial aflatoxin gene <i>Ver1</i>	[22]
Ver1-R	AGAGCTGGTCAGGATAATCCG			
verA-F	AACTCAATTGCACCACCAAA	898	The primer pair for amplification of partial aflatoxin gene <i>ver A</i>	[22]
verA-R	GAAGTAGTGGCCGGGTGAAT			
ordA-F	TATTCTGGGCGAAGCATCAA	1,156	The primer pair for amplification of partial aflatoxin gene <i>ord A</i>	[22]
ordA-R	CAGAGTAGTTGGTCCCACGA			
omtA-F	CAGGATATCATTGTGGACGG	594	The primer pair for amplification of partial aflatoxin gene <i>omt A</i>	[36]
omtA-R	CTCCTCTACCAGTGGCTTCG			
hypA-F	GCATGTCCGTCGTCCTGATA	654	The primer pair for amplification of partial aflatoxin gene <i>hyp A</i>	[22]
hypA-R	CCCATTGATCAATCTCGGAT			
glcA-F	AAGACACAGTCATCGCCTGTT	574	The primer pair for amplification of partial aflatoxin gene <i>glc A</i>	[22]
glcA-R	ACGCCTTTATCGAGCCAATA			

484

Table 3 Primers and resulting data from RAPD analysis

Primer code	Sequence (5'-3')	Number of amplified bands	Number of polymorphic bands	Percent polymorphic bands
RFu1	CCTGGGCCAG	9	5	55
RFu2	CCTGGGCGAG	21	3	61
RFu3	CCTGGGCTGG	20	18	90
RFu4	CCTGGGCTAT	7	7	100
RFu5	CCTGGGCTTG	10	7	70
RFu6	CCTGGGCTAC	16	16	100
RFu7	CCTGGGCTTA	14	14	100
RFu8	CCTGGGTCGA	16	12	75
RFu9	CCTGGGTGCA	18	15	83
RFu10	CCTGGCTTGC	11	9	81
RFu11	CCTGGCTTAC	8	8	100
RFu12	CCTGGGTTAC	6	5	83
RFu13	CGGGGGATGG	11	11	100
RFu14	CTCCCTGACC	11	11	100
RFu15	GAGCACCTGT	12	10	83
RFu16	GAGCACGTCA	10	9	90
RFu17	GAGCACGGCA	7	5	71
RFu18	AGGCACGGAG	12	12	100
RFu19	GGCACCATTC	11	9	81
RFu20	GAGGGCATGT	4	4	100
RFu21	CCGGCCCCAA	7	6	85
RFu22	CCGGCCTTAA	10	9	90
RFu23	CCGGCCATAC	11	10	90
RFu24	CCGGCCTTCC	13	12	92
RFu25	CCGGCTGGAA	10	9	90

Table 4 Primers and resulting data from MP-PCR analysis

Primer code	Sequence (5'-3')	G+C content (%)	Annealing temperature (°C)	Number of amplified bands	Number of Polymorphic bands	Percent polymorphic bands
N1	GTGGTGGTGGTGGTG	67	55	12	8	66
N2	GACAGACAGACAGACA	50	55	14	14	100
UBC809	AGAGAGAGAGAGAGAGG	53	50	14	12	85
UBC810	GAGAGAGAGAGAGAGAT	47	52	10	8	80
UBC811	GAGAGAGAGAGAGAGAC	53	50	8	7	87
UBC812	GAGAGAGAGAGAGAGAA	47	55	16	8	50
ISSR-02	ACTGACTGACTGACTG	50	45	15	11	73
ISSR-10	CACCACCACCACCAC	67	51	12	12	100
AF80820	AGAGAGAGAGAGAGAGT	47	52	12	10	83
AF80821	AGAGAGAGAGAGAGAGC	53	57	7	7	100

Table 5 Data obtained from greenhouse study using non-aflatoxigenic *A. flavus* as biocompetitor

Treatment	Rhizospheric and geocarpospheric soil			Seeds		
	Non-aflatoxigenic strain	Aflatoxigenic strain	Total aflatoxin (ppb)	Seed-borne <i>Aspergillus fl avus</i> incidence (%) [€]	Proportion of atoxigenic isolates	Total aflatoxin (ppb)
Negative control ^Φ	0 ± 0	0 ± 0	0 ± 0	0 ± 0 ^a	0.0	0 ± 0
AFG1 (Positive Control)	0 ± 0	5.32 ± 0.17	18 ± 0.57	09 ± 0.57 ^b	0.0	14 ± 0.51
AFG15	5.07 ± 0.10	5.08 ± 0.02	08 ± 0.28	09 ± 0.86 ^b	58.4	06 ± 0.23
AFG20	4.73 ± 0.00	5.26 ± 0.00	06 ± 0.46	08 ± 1.15 ^b	18.7	10 ± 0.17
AFG26	5.08 ± 0.03	4.97 ± 0.00	14 ± 0.51	07 ± 0.57 ^b	71.5	04 ± 0.28
AFG48	5.19 ± 0.00	4.70 ± 0.01	09 ± 0.23	07 ± 0.28 ^b	92.9	01 ± 0.17
AFG64	4.88 ± 0.02	4.95 ± 0.00	11 ± 0.57	08 ± 0.57 ^b	25.0	08 ± 0.51
AFG65	4.96 ± 0.03	5.07 ± 0.09	07 ± 0.28	09 ± 1.15 ^b	61.1	04 ± 0.57
AFG71	4.93 ± 0.01	5.14 ± 0.16	10 ± 0.51	09 ± 1.15 ^b	16.7	09 ± 0.46
AFGS5	5.47 ± 0.00	3.3 ± 0.08	04 ± 0.17	10 ± 1.15 ^b	87.5	02 ± 0.11
AFGS12	5.54 ± 0.01	3.99 ± 0.00	05 ± 0.23	09 ± 0.57 ^b	94.5	00 ± 0.00
NRRL32353	4.78 ± 0.00	5.26 ± 0.01	11 ± 1.15	10 ± 0.86 ^b	20.0	09 ± 0.57
NRRL32354	4.80 ± 0.11	5.21 ± 0.00	13 ± 1.15	11 ± 1.15 ^b	25.0	07 ± 0.57
CV (%)	0.45	0.33	0.53	0.33	0.85	0.76

^Φ Seedlings are grown in pots without any fungal inoculation. After incubation period all *A. flavus* strains isolated were found producing aflatoxin when analyzed through icELISA. [€] Values in the parenthesis indicates the % of seed-borne non-aflatoxigenic fungal incidence. CFU: Colony forming units. [€]Incidence of strain in Log10 cfu g⁻¹ soil after four month incubation period. Values are the mean ±SE, CV: Coefficient of Variance, Means are significantly different at *P* < 0.05.

Fig. 1 Schematic diagram showing deletion pattern designations, based on thirteen genes from the aflatoxin biosynthetic pathway, in several non-aflatoxigenic strains and a single aflatoxigenic strain (AFG1) of *A. flavus*. Solid circles indicate positive PCR products, while open circles indicate negative PCR products for each examined gene. C1 and C3 are sugar utilization genes located in a flanking region of the AF gene cluster.

Fig. 2 Dendrogram showing genetic diversity among sampled *A. flavus* strains. It was inferred through unweighted pair-group method with arithmetic average (UPGMA) analysis and based on combined RAPD and MP-PCR data.