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*Full Length Research Paper*

# Aflatoxin $B_1$ producing potential of *Aspergillus flavus* strains isolated from stored rice grains

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**Aflatoxin $B_1$ (AFB1) producing potential of different strains of *Aspergillus flavus,* isolated from 1,200 stored rice grains collected from 43 locations in 20 rice growing states in India was investigated. Eighty-five strains of *A. flavus* were isolated from the discolored rice grains and tested for their AFB1 producing potential on different agar media. Among these, 43 strains were identified as AFB1 producers (ranging 0.2 – 40 µg/g agar). All the 43 strains of *A. flavus* produced AFB1 on yeast extract sucrose agar media (YES). However, 65% of the strains produced AFB1 on Czapek's agar, 53% of the strains on potato dextrose agar (PDA) and none of the strains on Aspergillus flavus and parasiticus agar media (AFPA). The strain, DRAf 009 produced maximum AFB1 (4.0 – 40 µg/g agar) on all the agar media tested. Five strains of *A. flavus* producing high amounts of AFB1 identified in agar media were evaluated for their AFB1 production on milled rice cultivars. The five strains produced AFB1 on all the rice cultivars. Out of 5 strains, the DRAf 009 produced maximum AFB1 (386 – 415 µg/g grain) on all the rice cultivars tested.**

**Key words:** Rice, *Aspergillus flavus*, AFB1.

## INTRODUCTION

Rice (*Oryza sativa* L.) is the most important staple food crop in India and the bulk of rice is grown in kharif or wet season. Frequent and heavy rainfall and floods particularly near to harvest in coastal areas in eastern, southern and western regions of the country, wet the crop and make panicles more prone to invasion by *Aspergillus* sp. (Reddy et al., 2004). In a preliminary study *A. flavus* isolates from rice grains were shown to possess the ability to produce AFB1 (Reddy et al., 2005). However, mycotoxin producing fungi is less commonly reported for rice than for many other cereal crops (Tanaka et al., 2007) but rice represents a very good substrate for fungal growth and toxinogenesis since it is used as an ideal culture medium to test the toxigenic potential of isolated strains (Bars and Bars, 1992). Among the aflatoxins, AFB1 is the most toxic form for mammals and presents hepatotoxic, teratogenic and mutagenic properties, causing damage such as toxic hepatitis, hemorrhage, edema, immunosuppression and hepatic carcinoma (Speijers and Speijers, 2004). It has been classified as a class 1 human carcinogen by the International Agency for Research on Cancer (IARC, 1993). Several disease outbreaks of aflatoxicosis in humans and animals have been reported due to the consumption of aflatoxin contaminated food and feed (Reddy and Raghavender, 2007). Fouzia and Samajpati (2000) isolated mycotoxin-producing fungi from contaminated grains of rice sold in the local markets of Calcutta, India. Waghray et al. (1988) reported *Aspergillus* sp. as the most predominant fungi in the grain samples of flood-affected paddy variety NLR 9672 collected from standing crop, threshing floors and storage sites in the Nellore district of Andhra Pradesh, India. Almeida et al. (1991) identified aflatoxigenic strains of *Aspergillus* in milled rice from different regions of Brazil. Jayaraman and Kalyanasundaram (1990) explored the rice bran for toxigenic mycoflora and reported that *A. flavus* as the major contaminant. Trung et al. (2001) had reported that

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**Table 1.** States and locations from where the seed was collected.

| S/N | State | Locations | No. of samples |
|---|---|---|---|
| 1 | Andhra Pradesh | 6 | 257 |
| 2 | Andaman and Nicobar | 1 | 34 |
| 3 | Assam | 3 | 40 |
| 4 | Bihar | 2 | 24 |
| 5 | Chattishgarh | 1 | 11 |
| 6 | Delhi | 1 | 122 |
| 7 | Gujarat | 2 | 12 |
| 8 | Jarkhand | 2 | 21 |
| 9 | Jammu & Kashmir | 2 | 27 |
| 10 | Karnataka | 4 | 92 |
| 11 | Kerala | 2 | 294 |
| 12 | Madhya Pradesh | 1 | 19 |
| 13 | Maharashtra | 1 | 7 |
| 14 | Meghalaya | 1 | 9 |
| 15 | Puducherry | 2 | 47 |
| 16 | Rajasthan | 1 | 10 |
| 17 | Tamil Nadu | 4 | 85 |
| 18 | Tripura | 2 | 11 |
| 19 | Uttaranchal | 1 | 28 |
| 20 | West Bengal | 4 | 50 |
| Total | | 43 | 1,200 |

*A. flavus* is the most predominant fungi in rice grains from South Vietnam.

To date comprehensive studies on aflatoxin production by the strains of *A. flavus* on different agar media and on native solid substrates (rice grains) are limited. Therefore, in our investigation, we have explored the production of AFB1 by different strains of *A. flavus* isolated from discolored rice grains in India.

## MATERIALS AND METHODS

### Rice seed sample collection

A total of 1200 discolored rice samples were collected from 43 locations in 20 rice growing states in India (Table1). The seeds collected were either from areas exposed to different weather conditions or stored at various storage conditions:

(1) Seeds from the crop exposed to heavy rains and floods.
(2) Seeds from the submerged or damp conditions.
(3) Seeds stored in the godown for 1 - 4 years.
(4) Seeds from the grain market.

To avoid the sampling error due to highly heterogeneous nature of fungal distribution, each 2 kg composite sample collected from one storehouse was a mixture of 10 sub-samples (200 g each). Such sub-samples were collected from five diagonal on each of the upper, middle and lower layers of each storehouse (Liu et al., 2006).

### Isolation of aflatoxin producing *A. flavus* from seed samples

Using agar plate method (ISTA, 1966), *A. flavus* was isolated from all the seed samples. Four hundred seeds from each sample were plated on one-half strength potato dextrose agar (PDA) medium containing Rose Bengal at 50 ppm (Cotty, 1994). The plates were incubated at room temperature and the presence of *A. flavus* was observed after 6 days. The *A. flavus* isolated from samples were further purified individually by sub culturing PDA slants. They were then identified according to Raper and Fennell (1965) and Klich (2002).

### Extraction of AFB1 from *A. flavus* strains grown on agar media

Eighty five strains of *A. flavus* isolated from rice grain samples were grown on sterilized different agar media (AFPA, Czapeks agar, PDA and YES agar) for 5 days at 25 ± 2°C. Three replications were maintained for each isolate for each media. AFB1 were extracted by grinding the moldy agar (20 g) in waring blender for 5 min with methanol (100 ml) containing 0.5% potassium chloride (KCl) (Lee, 1965). The mixture was filtered through Whatman No.1 filter paper and the clarified filtrate was concentrated in vacuum on a rotary evaporator to 1 ml and identified on thin layer silica gel chromatography (TLC) for presence and absence of AFB1. Then the dried residue was dissolved in minimum quantity of methanol and estimated by indirect competitive ELISA.

### Preparation of spore suspension

Five strains of high AFB1 producing *A. flavus* on agar media (DRAf

002, DRAf 006, DRAf 009, DRAf 012 and DRAf 018) isolated from rice grains and maintained on potato dextrose agar slants at 4°C were harvested by adding 10 ml of sterile distilled water to give a final concentration of approximately 2 x $10^{12}$ spores/ml.

#### Extraction of AFB1 from *A. flavus* strains grown on rice grains

The high aflatoxin producing strains in agar medium was used for this study. Polished milled rice grains from cultivars Koshihikari (*japonica*), Mugad Suganda (aromatic and scented *indica*), Pusa Basmati and Rasi (non-scented *indica*) were soaked 2 h in tap water (25 ml/50 g grain) and were sterilized by autoclaving. Fermentation was carried out in 500 ml Erlenmeyer flasks containing 50 g of rice inoculated with 1 ml of spore suspension (2 x $10^{12}$ cfu/ml) of five isolates of *A. flavus* for five days at 25 ± 2°C. Five isolates of *A. flavus* (DRAf 002, DRAf 006, DRAf 009, DRAf 012 and DRAf 018) from different samples collected across the country were used and for each isolate six replications were maintained. Grains with fungal growth (20 g) from individual cultures were blended in waring blender for 5 min with methanol (100 ml) containing 0.5% KCl (Reddy et al., 2009). The mixture was filtered through Whatman No.1 filter paper and the clarified filtrate was concentrated in vacuum on a rotary evaporator to 1 ml and identified on thin layer silica gel chromatography (TLC) for presence and absence of AFB1. Then the dried residue was dissolved in a minimum quantity of methanol and estimated by indirect competitive ELISA.

#### Identification of AFB1 on TLC

Aflatoxin production was evaluated by standard TLC to confirm the presence or absence of AFB1. Two replicates were analyzed by spotting crude extract of aflatoxins. The TLC plates used were coated with silica gel 60 F254 on aluminum sheet, 20 x 20 cm and developed in chloroform/methanol (97:3) (Reddy et al., 2004). The plates were then observed under UV light at 365 nm and compared with standard aflatoxin spotted on the same plate.

#### Materials for ELISA

AFB1, AFB1-bovine serum albumin (BSA) conjugate, goat anti-rabbit IgG-ALP conjugate, *ρ*-nitrophenyl phosphate and BSA used were from Sigma, St Louis, USA, and microtiter plates (Maxi-sorp F96) were from Nunc (Nalge Nunc International, Denmark). All other chemicals were of reagent grade or chemically pure. Highly specific polyclonal antibodies for AFB1 were purchased from International Crop Research Institute for the Semi Arid Tropics, Patancheru, India (Devi et al., 1999).

#### Estimation of AFB1 by ELISA

The AFB1 was estimated by the method of Reddy et al. (2009). Microtiter plates were coated with 100 ng/ml of AFB1-BSA in sodium carbonate buffer, pH 9.6 (150 µl/well) and left overnight at 4°C. They were then washed in PBST, added with BSA (0.2%) and allowed to stand at 37°C for 1 h. ELISA plates were again washed with PBST and added with 100 µl AFB1 standards ranging from 25 ng to 10 pg/ml. Pre-incubation was carried out with 50 µl antiserum diluted in PBST-BSA (1:6000) and held for 45 min at 37°C. Filtrate samples extracted from rice grains and agar media with aqueous methanol-KCl as described earlier were added to wells at 1:10 dilution in PBST-BSA, that is, 100 µl/well. Goat antirabbit immunoglobulins conjugate to alkaline phosphatase were used at a 1:4000 dilution to detect rabbit antibodies attached to AFB1-BSA. *ρ*-nitrophenyl phosphate was used as a substrate at 0.5 mg/ml. Absorbance was recorded at 405 nm with an ELISA plate reader (Bio-Rad-680) after incubation at 28°C for 45 - 60 min. Standard curves were obtained by plotting log10 values of AFB1 dilutions at A405. The AFB1 (ng/ml) in samples was determined from the standard curves as: AFB1 µg/kg of agar or milled rice = [aflatoxin (ng/ml) in sample x buffer (ml) x extraction solvent (ml)]/sample weight (g). In order to test the recovery of AFB1, 20 g healthy rice grain was mixed with pure AFB1 (Sigma, St Louis, USA) to give concentrations ranging from 5 to 100 µg/kg. Rice samples were extracted and assayed as for unknown samples.

## RESULTS AND DISCUSSION

### AFB1 production by *A. flavus* isolates on agar media

Out of 85 strains of *A. flavus* tested, 43 strains produced AFB1 on TLC compared with standard at $R_f$ 0.47. Other strains of *A. flavus* did not produce any toxin on any media tested. The results on production of AFB1 by the strains of *A. flavus* through ELISA revealed that the capacity to produce was in the range of 0.2 - 40 µg/g of agar. All the strains of *A. flavus* (43) produced AFB1 on YES media and none of the strains on AFPA. Isolates from Tamil Nadu and Maharashtra produced high AFB1 on agar media. One isolate of *A. flavus* (DRAf 009) from Tamil Nadu produced maximum amount of AFB1 (40 µg/g) at 25°C, on YES agar media (Table 2). Sixty-five percent of the isolates produced AFB1 on Czapeks agar and 53% of the isolates on PDA agar media.

Fente et al. (2001) evaluated different agar (Czapeks, YES, coconut agar, aflatoxin producing ability (APA) media, coconut extract agar and coconut cream agar) media for aflatoxin production by *Aspergillus* sp. They reported more aflatoxin production in YES media compared to others. In our investigation we also found that YES agar media is the best media for AFB1 production by *A. flavus*.

Manisha and Sandip (2003) isolated *A. flavus* strains from rice mill surrounding air and tested aflatoxin producing capability in Czapeks agar, APA media and CAM agar. They found high aflatoxin production in Czapeks media produced by *A. flavus* compared to other media. In this study 65% of the *A. flavus* strains produced AFB1 on Czapeks agar. Carballo and Miguel (1987) collected 133 samples (mixed feeds and cereal grains) were examined for the incidence of strains of the *A. flavus* group. They tested the ability to produce aflatoxin in all strains isolated on cracked rice, APA medium and glucose-yeast extract agar (GYA) medium. Ten of the 67 isolates of *A. flavus* were aflatoxin-producing strains in rice and GYA medium; only three were aflatoxin-positive on the APA test.

### AFB1 production by *A. flavus* isolates on rice grains

Five strains of highest AFB1 producing *A. flavus* strains, identified on agar media were evaluated for their potential of AFB1 production on different rice cultivars. All the 5 strains produced AFB1 ranging from 9-415 µg/g of grains on all the rice cultivars tested (Table 3). Among the five

**Table 2.** Production of AFB1 (µg/g agar) by *A. flavus* strains isolated from stored rice grains on agar media at 25±2°C.

| Isolates | Place of collection | Different agar media and AFB1 (µg/g agar) | | | |
|---|---|---|---|---|---|
| | | Czapeks | YES | PDA | AFPA |
| DRAf 002 | Tamil Nadu | 0.5 | 6.4 | - | - |
| DRAf 006 | Tamil Nadu | 2.0 | 4.5 | - | - |
| DRAf 007 | Andhra Pradesh | 0.5 | 1.0 | 0.5 | - |
| DRAf 009 | Tamil Nadu | 4.0 | 40.0 | 4.0 | - |
| DRAf 010 | Jammu & Kashmir | 0.5 | 2.0 | 1.0 | - |
| DRAf 011 | Bihar | 1.0 | 1.0 | - | - |
| DRAf 012 | Maharashtra | - | 5.8 | - | - |
| DRAf 013 | Delhi | 1.0 | 0.5 | 1.0 | - |
| DRAf 018 | Maharashtra | 1.0 | 5.0 | 2.0 | - |
| DRAf 019 | Pondicherry | 2.0 | 0.5 | 2.0 | - |
| DRAf 022 | Assam | - | 1.5 | - | - |
| DRAf 026 | Karnataka | - | 2.0 | 1.5 | - |
| DRAf 027 | Andhra Pradesh | 0.5 | 0.2 | - | - |
| DRAf 029 | Tamil Nadu | - | 1.5 | 0.5 | - |
| DRAf 030 | Maharashtra | 1.0 | 3.0 | 2.0 | - |
| DRAf 031 | Chattishgarh | - | 1.5 | 1.5 | - |
| DRAf 032 | Pondicherry | 0.5 | 0.5 | 3.0 | - |
| DRAf 033 | Jarkhand | 1.0 | 0.5 | - | - |
| DRAf 038 | Tamil Nadu | - | 2.0 | - | - |
| DRAf 039 | Tripura | 0.5 | 2.5 | 1.0 | - |
| DRAf 040 | Andhra Pradesh | 2.0 | 0.5 | - | - |
| DRAf 042 | West Bengal | - | 1.0 | 1.5 | - |
| DRAf 043 | Chattishgarh | 0.2 | 1.0 | 1.0 | - |
| DRAf 044 | Tamil Nadu | 1.5 | 0.5 | - | - |
| DRAf 046 | Rajasthan | 2.0 | 0.5 | - | - |
| DRAf 047 | Madhya Pradesh | - | 2.0 | 1.5 | - |
| DRAf 048 | Kerala | - | 1.0 | 1.0 | - |
| DRAf 049 | Jarkhand | - | 2.5 | - | - |
| DRAf 050 | Uttaranchal | 0.5 | 0.2 | 1.5 | - |
| DRAf 051 | Andhra Pradesh | 2.0 | 1.5 | - | - |
| DRAf 052 | West Bengal | - | 1.0 | 0.5 | - |
| DRAf 055 | Tamil Nadu | 1.0 | 0.5 | 0.5 | - |
| DRAf 056 | Gujarat | 1.0 | 0.5 | - | - |
| DRAf 057 | Kerala | - | 1.0 | - | - |
| DRAf 059 | Maharashtra | 2.0 | 1.0 | 1.0 | - |
| DRAf 062 | Pondicherry | 1.0 | 1.5 | - | - |
| DRAf 065 | Tamil Nadu | 0.5 | 2.5 | 0.5 | - |
| DRAf 068 | Andhra Pradesh | - | 3.0 | - | - |
| DRAf 070 | Rajasthan | 1.0 | 1.0 | 0.5 | - |
| DRAf 072 | Bihar | 0.5 | 0.2 | - | - |
| DRAf 078 | Tripura | - | 1.0 | 0.5 | - |
| DRAf 080 | Tamil Nadu | 1.0 | 0.5 | - | - |
| DRAf 081 | Meghalaya | - | 1.5 | - | - |
| CD (*P*=0.05) | | 0.9 | 7.4 | 0.6 | - |
| CV (%) | | 18.5 | 26.8 | 15.8 | - |

Czapeks, Czapeks agar; YES, yeast extract sucrose agar; PDA, potato dextrose agar; AFPA, Aspergillus flavus and parasiticus agar; - = no toxin.

**Table 3.** Production of AFB1 (µg/g rice grain) by *A. flavus* strains isolated from stored rice grains on rice kernels.

| Isolate | Place of collection | Rice kernels and AFB1 (µg/g rice grain) | | | |
|---|---|---|---|---|---|
| | | Koshihikari | Mugad Suganda | Pusa Basmati 1 | Rasi |
| DRAf 002 | Tamil Nadu | 54 | 64 | 62 | 60 |
| DRAf 006 | Tamil Nadu | 11 | 11 | 13 | 9 |
| DRAf 009 | Tamil Nadu | 386 | 405 | 415 | 392 |
| DRAf 012 | Maharashtra | 12 | 11 | 15 | 9 |
| DRAf 018 | Maharashtra | 31 | 33 | 36 | 28 |
| CD (*P*=0.05) | | 8.6 | 6.4 | 10.2 | 4.8 |
| CV (%) | | 1.8 | 3.2 | 1.4 | 1.2 |

strains, DRAf 009 produced maximum AFB1 on four rice cultivars (range 386-415 µg/g of grains). The aflatoxin production capacity of 13 aflatoxigenic moulds isolated from natural black olive samples were reported to vary from 0.11 to 5.9 µg/g on rice substrate (Eltem, 1996). Begum et al. (1994) had reported the production of aflatoxin by *A. flavus* at 10.4 mg/kg rice substrate. Demet et al. (1995) studied the production of AFB1 at different intervals by artificial inoculation of *A. parasiticus* on rice grains. They reported a more or less constant le-vel of 30 mg AFB1/kg of rice grains. Aflatoxin production was estimated in 11 cultivars of rice and 6 cultivars of wheat following inoculation of seed with *A. parasiticus*. A marked variation was found in the amounts of AFB1 and AFG1 produced by the different cultivars. In general wheat grain was a poor source of the toxin compared with rice grain (Sinha et al., 1991). Refai et al. (1993) had reported that AFB1 production by *A. flavus* on rice, maize and YES media at 52, 40 and 40 µg/50 g substrate, respectively. Nandi and Haggblom (1984) reported aflatoxin production on rough rice grains (2430 - 10,643 µg/kg) by inoculating the grains with *A. flavus*. The total yield of aflatoxin production in culture by *A. flavus* isolate from peanut was 1511 µg/g of polished rice substrate in 5 days in shake cultures at 28°C (Shotwell et al., 1966). But the maximum amount of aflatoxin production estimated in this investigation was only 415 µg/g of substrate used at 25°C in still culture.

## Recovery of AFB1 from rice samples

The effectiveness of the extraction procedure was confirmed by adding pure AFB1 to ground rice and extracted in methanol-KCl. Recoveries from rice samples estimated by ELISA were greater than 95%.

## Conclusion

In this study, neither AFB2 nor AFG1 was detected and the bulk of aflatoxins produced by *A. flavus* isolates from stored rice grains belonged to AFB1 only on different agar media and solid substrates. It is therefore, apparent that *A. flavus* in tropical rice mostly produce AFB1. Bulk production of AFB1 on rice grains by high aflatoxin producing *A. flavus* strains will help to generate pure standards at an economical price and to produce antibodies for ELISA detection and estimation of AFB1 contamination in rice intended for domestic and export markets.

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