

Screening of Seed-Borne *Aspergillus flavus* Isolates for Aflatoxin Production in Pulses

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Abstract:

Mycotoxins, especially the group called aflatoxins, are small molecules toxic to both humans and animals. They are produced by two fungi, *Aspergillus flavus* & *A. parasiticus*. In pulses, *A. flavus* is dominant fungi which produce aflatoxins. *A. flavus* is the most common species in Africa and Asia. aflatoxins in seeds make them unpalatable to herbivores. They also show inhibitory effect on seed germination. Aflatoxins are potent toxic, carcinogenic, mutagenic, immunosuppressive agents, produced as secondary metabolites by *A. flavus*.

To study aflatoxins in pulses seeds, the different isolates of *Aspergillus flavus* were grown on liquid medium by phytotoxin production method. The analysis of aflatoxins was done by using TLC method (Jones, 1972). Totally fifteen isolates of *A. flavus* isolated from five different pulses seed crops were screened for their efficiency to produce aflatoxin on substrate (seed meal) and non-substrate GN medium. It is clear that the results regarding production of aflatoxin by different isolates of *A. flavus* that though majority of isolates of *A. flavus* showed positive test for aflatoxigenic nature, they also varied in their degree of aflatoxin production. This clearly suggests existence of aflatoxigenic strains in *A. flavus*.

The results regarding aflatoxin production on different substrates gives an idea that *A. flavus* might be very much specific for quality of substrate for aflatoxin production, as seed meal of gram and pigeon pea supported maximum aflatoxin production while, that of black gram proved inferior.

Keywords: Isolates of *A. flavus*, Phytotoxin method & TLC method.

Introduction:

Pulses constitute an important component of the Indian food system and play a major role in nutritional security because of their high content of proteins, dietary fibre, minerals, vitamins and other bioactive compounds. India is one of the major pulse-producing and pulse-consuming countries in the world, with chickpea (*Cicer arietinum* L.), pigeon pea (*Cajanus cajan* (L.) Millsp.), lentil (*Lens culinaris* Medik.), mung bean (*Vigna radiata* (L.) R. Wilczek), urd bean (*Vigna mungo* (L.) Hepper), field pea (*Pisum sativum* L.) and other grain legumes constituting important components of Indian agriculture and diets. Pulses are particularly important in India because they provide an economical source of plant protein and complement cereal-based diets. Therefore, maintaining the physical, nutritional and microbiological quality of pulse seeds from production through storage and consumption is essential for food and nutritional security.

However, pulse seeds are vulnerable to deterioration caused by several biotic and abiotic factors during harvesting, drying, transportation and storage. Fungi associated with seeds may adversely affect seed germination, color, nutritional quality and storage stability and may also cause biochemical deterioration. Under favorable environmental conditions, certain fungi can produce secondary metabolites known as mycotoxins, which constitute an important food-safety concern. Inadequate drying, high seed moisture, elevated relative humidity, poor ventilation and improper storage practices can favour fungal development and mycotoxin accumulation. In India, warm and humid climatic conditions, together with intermittent rainfall and inadequate post-harvest drying and storage, can create favorable conditions for the growth of toxigenic fungi (FAO, 2004).

Among the fungi associated with stored agricultural commodities, *Aspergillus flavus* Link is of particular importance because of its ability to produce aflatoxins. The fungus may occur in soil, plant residues and agricultural

commodities and can colonize seeds both before harvest and during storage. Aflatoxins are secondary metabolites produced mainly by toxigenic strains of *A. flavus* and related species. The principal naturally occurring aflatoxins are aflatoxin B₁ (AFB₁), B₂ (AFB₂), G₁ (AFG₁) and G₂ (AFG₂). Among these, AFB₁ is considered the most potent and toxicologically significant aflatoxin. Aflatoxins are of major public-health concern because exposure may cause acute toxicity, immunosuppression, growth impairment and chronic liver damage, while prolonged exposure to AFB₁ is strongly associated with hepatocellular carcinoma (IARC, 1993; WHO, 2024). The International Agency for Research on Cancer has classified naturally occurring aflatoxin mixtures as carcinogenic to humans, highlighting the importance of controlling aflatoxin contamination in food commodities (IARC, 1993).

The occurrence of *A. flavus* and aflatoxins in Indian agricultural commodities has been documented for several decades. Maggon et al. (1969), while examining Indian strains of *A. flavus* isolated from Delhi soils, demonstrated considerable variation in aflatoxin-producing ability among isolates. Of the nine *A. flavus* strains examined, seven produced aflatoxins B₁ and B₂, whereas the remaining isolates did not produce detectable aflatoxins under the conditions tested. This early observation established an important principle that remains relevant to aflatoxin research: the presence of *A. flavus* does not necessarily indicate aflatoxin production, because toxigenicity varies among individual isolates.

The association of aflatoxin-producing fungi with pulses and other foodgrains has subsequently been demonstrated in India. In a study of food commodities available in local markets, Roy et al. (2001) isolated mycotoxin-producing fungi from rice, pulses and oilseeds and demonstrated AFB₁ production by *A. flavus* and *A. parasiticus*. The study reported substantial variation in AFB₁ production among fungal isolates and commodities, confirming that pulses can serve as suitable substrates for aflatoxin-producing fungi.

Reddy et al. (2011) further investigated the AFB₁-producing potential of five *A. flavus* strains on several commercially important food grains, including green gram and horse gram. All five isolates produced AFB₁ under the experimental conditions, but the amount of toxin produced varied considerably among isolates and food substrates. AFB₁ accumulation in green gram ranged from 645.8 to 2,245.2 µg kg⁻¹. These findings demonstrate that aflatoxin production is influenced not only by the presence of *A. flavus*, but also by the particular fungal strain and the commodity on which it develops.

Studies conducted on seed-borne fungal contamination have provided further evidence of the importance of *A. flavus* in Indian food and seed systems. Investigations of stored seeds and foodgrains have demonstrated that *A. flavus* can be a predominant fungal contaminant and that its isolates may differ in aflatoxigenic potential. Importantly, studies conducted on Indian sorghum seed samples showed that the incidence of *A. flavus* was not necessarily correlated with aflatoxin concentration. Among 37 *A. flavus* strains isolated, six were found to be non-aflatoxigenic when assessed using cultural, thin-layer chromatography and immunoassay approaches (Kumar et al., 2014). This observation is particularly relevant to seed-health investigations because it demonstrates that fungal incidence alone cannot be used as a reliable indicator of aflatoxin risk.

Environmental conditions have a major influence on both fungal colonization and aflatoxin biosynthesis. Availability of moisture, temperature, relative humidity, aeration and substrate composition are among the principal factors regulating the growth of *A. flavus* and subsequent aflatoxin production. In India, warm and humid climatic conditions can favor fungal invasion and toxin production, particularly when harvested seeds are not adequately dried before storage. The Food and Agriculture Organization has identified grain moisture or the relative humidity surrounding the commodity as important determinants of *A. flavus* growth and aflatoxin production and has recognized several Indian agro-climatic regions as potentially conducive to mycotoxin development (FAO, 2004).

The interaction between fungal strain and environmental conditions is particularly important because conditions that support fungal growth do not always result in equivalent levels of aflatoxin production. Aflatoxin biosynthesis is a complex, strain-dependent process influenced by temperature, water activity, nutrient availability and other environmental factors. Consequently, isolates belonging to the same fungal species and recovered from the same

commodity may exhibit markedly different toxigenic capacities. Experimental studies have also shown that different foodgrains and pulses may support different levels of AFB₁ accumulation even when inoculated with the same *A. flavus* strain (Reddy et al., 2011).

Another important consideration is that the detection of fungal growth on seeds and the detection of aflatoxin are two different parameters. Visible fungal colonization may not always correspond to toxin production, and aflatoxin contamination may occur without extensive visible mould development. Therefore, determining the toxigenic potential of individual *A. flavus* isolates provides more meaningful information than merely recording the incidence of the fungus on seeds. Screening isolates for aflatoxin production can help distinguish toxigenic and non-toxigenic populations and can provide an indication of the potential food-safety risk associated with contaminated pulse lots.

The identification and characterization of toxigenic *A. flavus* isolates are also important from the perspective of developing effective strategies for aflatoxin management. Knowledge of the occurrence and toxigenic potential of seed-borne isolates can assist in identifying high-risk commodities, improving post-harvest handling and storage practices, developing appropriate seed-treatment strategies and supporting the selection of non-aflatoxigenic strains for biological control programs. The existence of naturally occurring non-aflatoxigenic *A. flavus* populations is particularly important because such strains may have potential for use in competitive exclusion-based biocontrol approaches, provided their efficacy and safety are adequately established.

In India, where pulses are widely cultivated, stored and consumed, systematic investigation of seed-associated toxigenic fungi is therefore important for ensuring the safety and quality of pulse-based foods. Although several studies have reported the occurrence of *A. flavus* and aflatoxin contamination in foodgrains, the toxigenic potential of individual seed-borne isolates may vary according to pulse species, geographical origin, environmental conditions and fungal genotype. Hence, assessment of *A. flavus* isolates at the individual strain level is necessary to obtain a clearer understanding of the aflatoxin-producing potential associated with pulse seeds.

In view of the nutritional importance of pulses, their widespread consumption in India and the potential health hazards associated with aflatoxin exposure, the present study was undertaken to screen seed-borne *Aspergillus flavus* isolates associated with pulses for their aflatoxin-producing ability. The study is expected to provide useful information on the occurrence and toxigenic potential of seed-borne *A. flavus* isolates and to contribute to the development of appropriate strategies for reducing aflatoxin-related risks in pulses intended for food and feed purposes.

The study of mycoflora associated with seeds of five pulses namely gram, pigeon pea, green gram, black gram & lentil reveals that *Aspergillus flavus* is one of the dominant fungi changing the biochemical composition of pulses seeds.

This change in biochemical composition of seed is due to toxin called aflatoxin. So the present investigation is studies aflatoxins production in selected pulses.

Material and methods:

a) Production of Phytotoxins:

The test fungi isolated from pulses seeds were grown on liquid medium containing glucose 1 %, KNO₃ 0.25 %, KH₂PO₄ 0.1 % and MgSO₄.7H₂O 0.05 %. 25 mL of the medium was added in 100 mL conical flask and autoclaved at 15 lb pressure for 15 min. On cooling, flasks were inoculated separately with one mL of spore suspension of test fungi prepared from 7 day old cultures grown on PDA slants. The flasks were incubated at 25 ± 2°C for nine days and were harvested by filtering their contents through Whatman filter paper No. 1. The filtrates were collected in presterilized culture bottles and termed as crude toxin preparations. The preparations were tested for their toxicity. (Mungikar *A.*, 1982)

b) Assay of Aflatoxins (TLC Method)

The analysis of aflatoxins was done by using the method of Jones (1972). 30 gm of silica gel a (with CaSO₄ as binder) was taken in a stoppered flasks and mixed with 60 mL of distilled water, the slurry was uniformly spread using applicator on a clean glass plate keeping the thickness of layer 0.25 mm. The dry plate was activated at 110⁰ C for 30 min. in hot air oven. The gel on plate was divided into number of lanes by drawing lines with a sharp needle.

The Chloroform extract of toxins were then spotted in known volume in various lanes carefully with micro capillary tube on an imaginary line 2.5 cm away from one end of the plate. The plate was developed in solvent system of toluene : ethyl acetate : formic acid (6 : 3: 1) in a chromatographic tank for 50 minutes till solvent reached up to 20 mm below the top end of the plate. Plate was dried at room temperature and the fluorescing spots of toxins were visualized with UV lamp in UV cabinet (360 nm). The blue fluorescent spots were identified as aflatoxin B₁ and B₂ on the basis of the intensity of the fluorescent spots.

Result and discussion:

Totally fifteen isolates of *A. flavus* isolated from five different pulses seed crops were screened for their efficiency to produce aflatoxin on substrate (seed meal) and non-substrate GN medium. The results are summarized in Table 01 and Fig. 1. Among three isolates of *A. flavus* from gram, GRAF – 1 gave maximum production of aflatoxin which was followed by isolate GRAF – 2. The isolate GRAF – 3 did not produce aflatoxin on seed meal but it has shown production on GN medium.

Among the three isolates of *A. flavus* from pigeon pea, isolates PPAF – 1 and PPAF – 2 were found to be highly aflatoxigenic in nature while, isolate PPAF – 3 produce more amount of aflatoxin on GN medium.

The three isolates of *A. flavus* from green gram seeds, there was no production of aflatoxin by GGAF – 3 on substrate medium. Similarly among two isolates of *A. flavus* from black gram seeds, isolate BGAF – 1 produced very low amount of aflatoxin.

Among three isolates of *A. flavus* from lentil, LAF – 1 gave maximum production of aflatoxin which was followed by isolate LAF – 2. The isolate LAF – 3 did not produce aflatoxin on seed meal but it has shown production on GN medium.

The significance of pulse-associated *A. flavus* has gained further attention in recent research. Comparative studies have demonstrated that pulse species can differ in their susceptibility to *A. flavus* infection and aflatoxin accumulation. Recent experimental work involving protein-rich pulses has shown that although pulses may accumulate lower levels of AFB₁ than some other commodities under particular experimental conditions, they can nevertheless support *A. flavus* infection and the accumulation of mycotoxins, emphasizing the need for crop-specific assessment of fungal and toxin risks (Branstad-Spates et al., 2025).

Table 01: Production of aflatoxin in different isolates of *Aspergillus flavus*.

Name of isolate with source	GN medium		Seed meal medium	
	B ₁	B ₂	B ₁	B ₂
Gram				
GRAF – 1	+++	+++	++	+
GRAF – 2	++	++	+	+
GRAF – 3	+	+	-	-
Pigeon pea				
PPAF – 1	++++	+++	++	+
PPAF – 2	+++	++++	+	+
PPAF – 3	++	++	-	+

Green gram				
GGAF – 1	+	+	+	-
GGAF – 2	++	+++	+	+
GGAF – 3	+	+	-	-
Black gram				
BGAF – 1	-	-	+	-
BGAF – 2	+	+++	-	+
Lentil				
LAF-1	+++	++	+	+
LAF-2	++	+	+	+
LAF-3	+	+	-	-

+ = Very poor, ++ = Poor, +++ = Moderate, ++++ = High,

+++++ = Luxuriant

Conclusion:

The present investigation demonstrated that seed-borne *Aspergillus flavus* isolates associated with pulse seeds possess variable aflatoxin-producing potential. A total of 15 isolates obtained from gram, pigeon pea, green gram, black gram and lentil were screened for aflatoxin production using GN medium and seed-meal medium. The results showed considerable variation in aflatoxin production among isolates, indicating that the presence of *A. flavus* on pulse seeds does not necessarily imply an identical level of aflatoxin-producing ability.

Among the isolates examined, GRAF-1 from gram, PPAF-1 and PPAF-2 from pigeon pea, and LAF-1 from lentil exhibited comparatively higher aflatoxin production. In contrast, BGAF-1 produced only a low level of aflatoxin on seed meal, while GRAF-3, GGAF-3 and LAF-3 did not produce detectable aflatoxin on seed-meal medium but showed production on GN medium. These observations clearly demonstrate the influence of both fungal strain and growth substrate on aflatoxin production.

The study further indicated that seed-meal substrates differed in their suitability for aflatoxin production. Gram and pigeon pea supported comparatively greater production, whereas black gram was relatively less favourable. The differences observed between GN medium and seed-meal medium also suggest that substrate composition plays an important role in determining the expression of aflatoxigenic potential.

Overall, the findings emphasize that screening individual *A. flavus* isolates is more informative than simply determining the occurrence of the fungus in pulse seeds. Identification of highly aflatoxigenic isolates is important for assessing potential food-safety risks and for developing appropriate strategies for safe seed handling, drying and storage. Further studies involving quantitative estimation of individual aflatoxins, characterization of toxigenic and non-toxigenic isolates, and evaluation of environmental factors influencing toxin production would strengthen the understanding and management of aflatoxin contamination in pulses.

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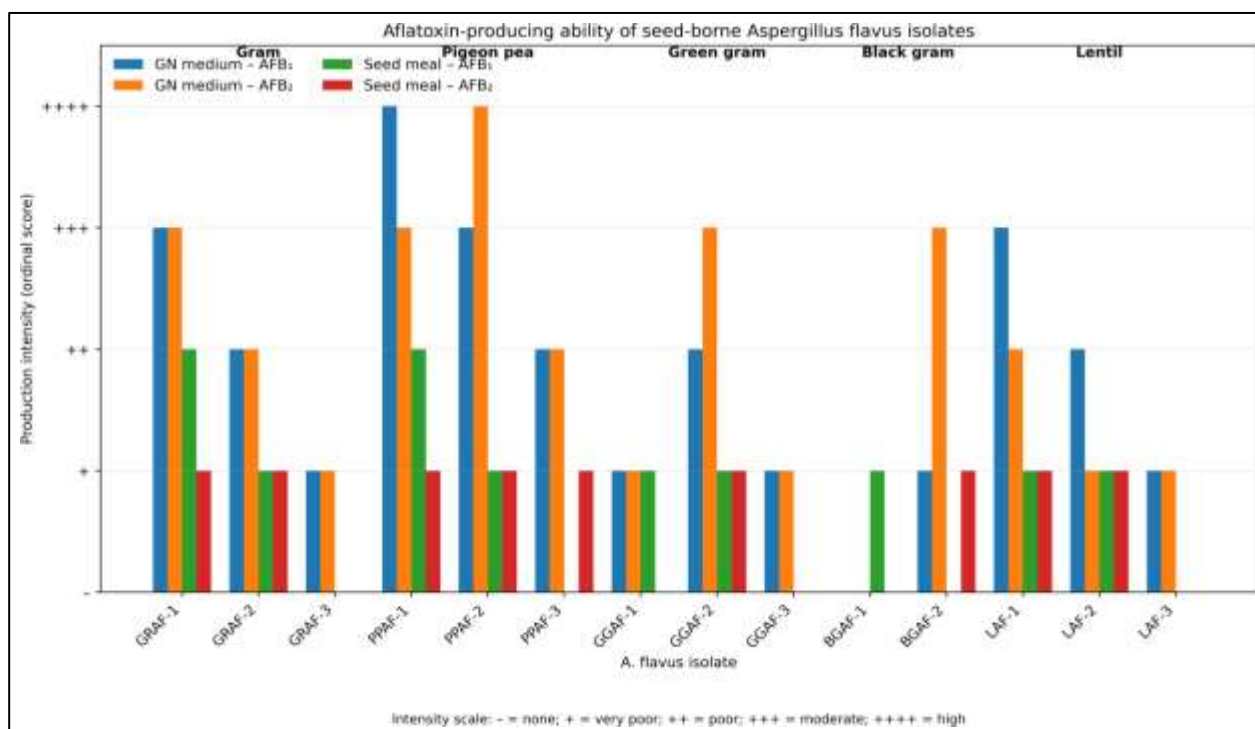


Fig. 01: Production of aflatoxin in different isolates of *Aspergillus flavus*.