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Inhibition of aflatoxin B_1 production by an antifungal component, eugenol in stored sorghum grains

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ABSTRACT

The potential use of antifungal component eugenol for the reduction of aflatoxin B_1 (AFB_1) in stored sorghum grain was investigated. Fungal infestation of sorghum results in deterioration of varied biochemical composition of the grain. In this study, three genotypes (M35-1; C-43; LPJ) were inoculated with two highly toxigenic strains of *Aspergillus flavus* with three different eugenol treatments in order to evaluate the AFB_1 production. From this study it was found that at 8.025 mg/g concentration, eugenol completely inhibited the AFB_1 production. The lowest amount of AFB_1 was observed in genotype M35-1, whereas higher amount AFB_1 was observed in LPJ followed by C-43. In all sorghum genotypes there was a significant positive correlation existing between protein content and aflatoxin produced, the *r* values being 0.789 and 0.653, respectively. Starch in three genotypes was found to have a significant negative correlation ($r = -0.704$; -0.609) with aflatoxin produced. The starch content decreased whereas the protein content in all sorghum varieties increased during infection.

1. Introduction

Antifungal chemicals were used for the preservation of stored grains (Paster, Menasherov, Ravid, & Juven, 1995). Due to health and economic considerations, natural plant extracts may provide an alternative method to protect food and feed from fungal contamination (Yin & Cheng, 1998). Eugenol (4-allyl-2-methoxy phenol), the active principle of cloves (*Syzygium aromaticum*) and of medicinal and aromatic plants, such as *Ocimum sanctum* and *Pimenta racemosa*, is generally used as a food flavoring agent (Jayashree & Subramanyam, 1999). In view of its non-mutagenic and non-carcinogenic properties (Anon, 1985), it is generally regarded as safe by the FAO (Opdyke, 1975) with an acceptable daily intake of up to 2.5 mg kg^{-1} body weight in humans (FAO, 1982). Clove is expensive, but eugenol can be commercially extracted from other sources such as *Ocimum gratissium*, *Ocimum tenuiflorum*, *O. sanctum*, *Cassia fistula*, *Zieria smitii*, *Clarkia breweri*, *Ageratum conyzoides* and whole tobacco, which are also known to be useful sources for eugenol (Reddy, Reddy, Prameela, Mangala, & Muralidharan, 2007).

Sorghum [*Sorghum bicolor* (L.) Moench] is one of the main staples for the world's poorest and most food insecure people. Sorghum is grown in *kharif* (rainy), *rabi* (post rainy) and summer seasons in India. Sorghum is a truly dual-purpose crop; both grain and stover are highly valued outputs. Grain mold on sorghum is an important disease worldwide, which causes considerable qualitative and quantitative damage (Audilakshmi et al., 2011). Grain molds cause significant losses in both grain yield and its nutritional quality. Sorghum is the preferred staple food in states of Maharashtra, Karnataka and Andhra Pradesh in India. Sorghum grain grown in *kharif* is severely affected by grain molds due to heavy rains at the time of crop maturity. If the extent of mold is severe the grain is unsafe for consumption due to the contamination of mycotoxins.

Grain sorghum is often damaged by rain in the field and severely infected by grain mold, which includes *Aspergillus* infection and aflatoxin production (Ratnavathi & Sashidhar, 2003). Mycotoxin contamination and grain mold of sorghum are considered as the most important constraints of grain quality and production, globally. Sorghum grains suffer from infection and colonization by several fungi during panicle and grain developmental stages (Waliyar et al., 2008, p. 32). Several species of *Aspergillus*, *Alternaria*, *Cladosporium*, *Diplodia*, *Fusarium*, *Curvularia*, *Phoma* and *Penicillium* are among the prevalent grain mold pathogens in sorghum (Bandyopadyay, Butler, Chandrashekar, Reddy, & Navi, 2000). Aflatoxins (AFs) are toxic secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Their presence in food is of great concern because of their harmful effects on human and animal health (Toteja et al., 2006). The

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most toxic among the AFs is AFB_1, which has been reported to be one of the most potent environmental carcinogens (Kuiper-Goodman, 1995; Montesano, Hainant, & Wild, 1997). The natural occurrence of aflatoxin B_1 in rain-affected Indian sorghum samples and aflatoxin B_1 contamination in Brazilian samples were reported by Silva, Pozzi, Mallozzi, Ortega, and Correa (2000). Sashidhar, Ramakrishna, and Bhat (1992) reported a systematic study on the mold and mycotoxin contamination in the grain sorghum stored in traditional containers in India. The global occurrence of mycotoxins is considered a major risk factor, and according to the Food and Agricultural Organization (FAO, 2004), 25% of the world's commodities are annually affected by known mycotoxins (Schatzmayr et al., 2006). Mycotoxin contamination is a serious concern that occurs in the field before harvest or during storage, despite efforts of prevention (Lillehoj, 1983). The improvement in sorghum grain quality occurs when the crop was harvested at physiological maturity and artificial drying (Audilakshimi et al., 2005). Grain mold resistance at physiological maturity is genetically governed and the grain mold score further gets compounded at harvest maturity depending on (environment) rainfall received after physiological maturity (Ambekar et al., 2011). Acetic acid treatment was most effective for reducing the grain mold score in sorghum (Audilakshimi et al., 2007).

Fungal deterioration of stored grains is a chronic problem in the Indian storage system because of the tropical hot and humid climate. Infection of *Aspergillus* spp. was found on most sorghum grains collected from different sorghum growing areas in India either at *kharif* season or during storage. Hence the present study was carried out on Inhibition of aflatoxin B_1 production by an antifungal component, eugenol on sorghum grains at different concentration levels and estimating the aflatoxin B_1 (μg/kg of grain sorghum) by Indirect competitive ELISA (Enzyme-linked Immunosorbent assay). It was explored in a four step approach in the laboratory conditions which are

1. Identification of highly toxigenic strains.
2. *In vitro* screening of sorghum cultivars with identified strains, to study the chemical quality of infested grains, through electron microscope.
3. Inhibition of AFB_1 production by an antifungal component, eugenol.
4. Chemical parameters of infested grain such as starch and protein were studied.

2. Materials and methods

2.1. Identification of highly toxigenic strains

The fungal strains used in this study were *A. flavus*, which were identified from sorghum samples. The procedure used for the isolation of the fungi was as follows: from each sample 25 seeds were randomly chosen and the surface disinfected with 1% NaOCl after which they were plated (10 seeds per plate) onto Potato Dextrose Agar and incubated at 30 °C for 7 days. Mycotoxin producing ability of 18 strains of *A. flavus* isolated from sorghum grain samples from different locations, were studied under laboratory conditions. Two different methods (Visual detection under UV light and Indirect ELISA) were used for detection and estimation of aflatoxins production by *A. flavus*.

2.2. Screening of sorghum cultivars

Grains from 15 released cultivars of sorghum (CSH 9, CSH 14, CSH 15R, CSH 17, CSH 18, CSH 19R, CSV 13, CSV 14R, CSV 15, CSV 18, CSV 19SS, CSV 216R, SPV 462, SPV 1430 and SPV 1616) were collected from *Rabi* 2005 season and used for the screening. The grains were inoculated with two highly toxic strains (A112 and A104) *in vitro* and allowed to grow at 28 °C for 12 days. These grains were dried at 40 °C and then processed for toxin extraction. The infested grains were cleaned & processed for electron microscopy for chemical quality.

2.3. Inhibition of AFB_1 production by an antifungal component, eugenol

2.3.1. Preparation of grain samples for eugenol treatment

A total of 3 sorghum varieties M35-1, C-43 and LPJ were collected for eugenol treatment. These varieties were multiplied at the farms of the Directorate of Sorghum Research (formerly National Research Centre for Sorghum), Rajendranagar, Hyderabad,

Table 1
Aflatoxin B_1 production by isolates of *Aspergillus flavus* from sorghum.

S. no	Name of *Aspergillus* isolate	Source of sorghum s ample	Location of collection	Colony color on Saboured agar[a]	Intensity of fluorescence (1–5 scale)	AFB_1 (μg/kg)[c]
1	A30	Market	Guntur	Dark green	3.0 (1.4)[b]	1371
2	A31	Market	Guntur	Yellowish green	2.0 (0.0)	59
3	A81	Market	Mahaboob Nagar	Dark green	3.0 (1.4)	−1
4	A112	Market	Mahaboob Nagar	Dark green	3.5 (0.7)	8003
5	A151	Market	Lingampally, Hyderabad	Yellowish green	3.0 (0.0)	−4
6	A103	Market	Samshabad, Hyderabad	Olive green	3.0 (0.7)	−1
7	A104	Market	Samshabad, Hyderabad	Olive green	4.5 (0.7)	5152
8	A60	Market	Malakpet, Hyderabad	Yellowish green	3.0 (1.4)	655
9	A64	Market	Malakpet, Hyderabad	Yellowish green	4.0 (0.0)	1900
10	A125	Market	Erragadda, Hyderabad	Dark green	3.5 (0.7)	2275
11	A133	Market	Erragadda, Hyderabad	Olive green	3.5 (2.1)	−1
12	A256	Market	Indore	Yellowish green	3.5 (0.7)	0
13	A41	Brewery	Mumbai	Dark green	3.0 (0.0)	1
14	A42	Brewery	Mumbai	Dark green	4.0 (0.0)	6766
15	A74	Brewery	Indore	Yellowish green	4.5 (0.7)	2865
16	A254	Field	Indore	Dark green	4.0 (1.4)	−2
17	A250	Field	Nalgonda	Yellowish green	3.5 (0.7)	8
18	A252	Field	Rajendranagar	Yellowish green	2.0 (0.0)	2
19	Control	–	–	–	–	−1

[a] Saboured dextrose amended with 0.3% β-cyd.
[b] Figures in the parenthesis are standard deviations.
[c] 20 ppb is the safety limit of aflatoxin as per the CODEX committee.