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Inhibition of *Aspergillus flavus* colonization and aflatoxin (AfB1) in peanut by methyleugenol

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Methyleugenol is naturally occurring substance in oils and fruits and in various foods as flavoring agent. Effect of this methyleugenol in inhibiting *A. flavus* colonization and aflatoxin production on peanut pods and kernels has been studied. Spray of methyleugenol (0.5 %) on peanut pods and kernels checked the colonization of *A. flavus* and aflatoxin synthesis. This chemical can be used as both prophylactic or post infection spray on peanut pods before storage. It is the first report on the inhibition of *A.flavus* by methyleugenol on peanut.

Keywords: Aflatoxin, *Aspergillus flavus*, Methyleugenol, Peanut pod, Peanut kernel

Aspergilli produce mycotoxins in food and feedstuffs. It has been reported that at least 25% of the grain produced worldwide each year get contaminated with mycotoxins[1]. Among mycotoxins, aflatoxins are known for carcinogenic, teratogenic and immunosuppressive secondary metabolites produced by *Aspergillus flavus* and *Aspergillus paraciticus*. *A. flavus* group of fungi invading peanut produces four types of aflatoxins,*viz.,* B1, B2, G1, G2 and of these B1 is the most toxic form followed by G1, B2 and G2.

Peanut (*Arachis hypogaea* L.) is the cash crop in the rain fed area in many Asian countries. Due to occurrence of pods in soil, peanut is the vulnerable crop for *A. flavus* especially at preharvest and subsequently at post harvest. Since, aflatoxins cause acute, subacute and chronic toxicity, currently about 100 nations have the regulations for aflatoxin limits in peanut and its products, which vary from 2µg/ kg to 200 mg/ kg.

Since, there is meager genetic tolerance and recommended cultural practices, which inhibit *A. flavus* growth and aflatoxin contamination in preharvest peanuts, efforts are being made to prevent this problem after harvest using new antifungal materials from natural sources and chemical preservatives[2,3]. Inhibitory effect on growth and aflatoxin production by Cinnamom and clove oils, which contain cinnamic aldehyde and eugenol have been reported earlier[4]. Since, clove oil is rich source of eugenol; its large scale usage is limited due to its exorbitant price. Methyleugenol (4-allyl 1, 2 dimethoxybengene) is naturally occurring substance present in many essential oils and fruits. It is colorless to pale yellow oily liquid soluble in water, ether and chloroform. It is used as a flavoring agent in jellies, baked goods, nonalcoholic beverages, chewing gum, candy, pudding, relish and ice cream at concentration from 0.002 to 0.3% (Ref. 5). It is safer to use as antifungal agent compared to available chemical pesticides or fungicides, which limit peanuts for human consumption. Methyleugenol is used in the present study to evaluate its effective inhibition of *A.flavus* colonization and aflatoxin production in pea nut.

Materials and Methods

Source of materials—Groundnut pods of variety K-134 was procured from ARS, Kadiri, Andhrapradesh. The toxic *Aspergillus flavus* was isolated from the native soils and the partial rDNA sequence was submitted to NCBI, USA (Acc.No-EF-030718.

Experiment I

Fungal growth inhibition assay—Antifungal activity of methyleugenol against *A.flavus* was determined by fungal growth inhibition assay as described earlier[6]. Extra pure (98.87%) methyleugenol

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(SRL, Mumbai) was mixed with PDA or 1 % of peanut meal agar [peanut kernel powder (1g) + 100 ml of agar] at different concentrations *viz*., 0.1, 0.5, 1.0 and 5.0 %. Disc (5 mm diameter) soaked in 5-day old *A. flavus* culture in potato dextrose broth medium was transferred at the center of the treated Petri dish and incubated at 28°±2°C for four days. Medium without methyleugenol served as control. After incubation for four days, the colony diameter was measured and inhibition percentage of the fungal growth as compare to control treatment was calculated.

Experiment II

Inhibition of pod and kernel infection—Peanut variety K-134 (susceptible variety) pods and kernels were surface sterilized by soaking in 0.1% aqueous solution of mercuric chloride for 3 min, rinsed with sterile distilled water and five pods and five kernels each were placed separately on filter paper in sterile Petri dish (10 cm diam). Six replicates with 5 pods and 5 kernels were maintained. These peanut pods and kernels were infected with virulent, toxigenic strain of *A. flavus*, by gently putting conidial spore suspension (1×10^6 spores per ml) on pods and kernel, so that inoculum get lodged on pod and kernel surface, then methyleugenol solution (1%) was added on pod and kernel gently and incubated at 25°C. To maintain high humidity, sterile distilled water (1-2 ml) was added everyday during the first five days. After six days of incubation, number of seeds contaminated by *A. flavus* was counted.

Experiment III

Prevention of pod and kernel infection—To verify the effect of methyleugenol in preventing *A.flavus* infection, peanut pods and kernels (4 each) were surface sterilized with 1% $HgCl_2$ and kept on filter paper in separate Petri dishes (10 cm diam; six replicates). Spread methyleugenol solution (1%) on surface of pods and kernels gently, and incubated at 28°C. After three days of incubation *A. flavus* (1×10^6 spores per ml) was spread on the surface of pods and kernels, incubated further at 28°C for 5 days. On day 6, number of pods and kernels contaminated by *A. flavus* was counted.

Experiment IV

Inhibition of infection in contaminated pod and kernel—To study the effect of methyleugenol in inhibiting the already contaminated peanut pods and kernel by *A. flavus*, peanut pods and kernels (4 each) were surface sterilized with $HgCl_2$ (1%) and kept on filter paper in sterilized Petri dishes (6 replicates). Then conidial spore suspension of *A.flavus* ($1x10^6$ spores per ml) was gently added at 28°C. On day 4, methyleugenol solution (1%) was spread on to the surface of pods and kernels and incubated further for 5 days. Number of pods and kernels contaminated by *A. flavus* was counted on day 6.

Estimation of aflatoxin B1 by indirect ELISA—Highly specific polyclonal antibodies for aflatoxins in indirect competitive ELISA procedure were[7] followed as per ICRISAT, Hyderabad, India. ELISA wells were coated with 100 ng/ml of aflatoxin B_1-BSA in sodium carbonate buffer, (*p*H 9.6; 150µl/well) and incubated for overnight at 4°C. They were then washed in PBST, (phosphate buffer saline tween) added with BSA (Bovine serum albumin) (0.2%) and allowed to stand at 37°C for l hr. ELISA plates were again washed with PBST and added aflatoxin B_1 (100 µl) 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 kept for 45 min at 37°C. Filtrate samples extracted from peanut kernels with aqueous 70% methanol-KCl as described earlier[8] were added to wells at 1:10 dilution in PBST-BAS. Goat antirabbit immunoglobulin conjugated to alkaline phosphatase were used at a 1:4000 dilution to detect rabbit antibodies attached to aflatoxin B_1-BSA. *p*-Nitro phenyl phosphate was used as a substrate at 0.5 mg/ml. Absorbance was recorded at 405 nm with an ELISA plate reader (Labsystem, 352) check after incubation at 28°C in dark for 45 min to 1hr. Standard curves were obtained by plotting log 10 values of aflatoxin B_1 dilutions at A405. Aflatoxin B_1 (ng/ml) in sample was determined from the standard curves as– [aflatoxin B_1 µg/kg of paddy or milled rice = (aflatoxin (ng/ml) in sample × buffer (ml) × extraction solvent (ml) / sample weight (g)].

Aflatoxin B_1, Aflatoxin B_1-BSA conjugate, goat anti rabbit IgG-ALP conjugate, *P*-nitro phenyl phosphate and bovine serum albumin (BSA) used were from Sigma chemical Co., St. Louis, USA, and microtitre plates (Maxi-sorp f 96) were from Nunc (Nalge Nunc International, Denmark). All other chemicals were reagent grade or chemically pure.

Results

Fungal growth inhibition assay—Methyleugenol (0.5%) inhibited *A. flavus* colonization completely (100%) on PDA and PDA having peanut substrate

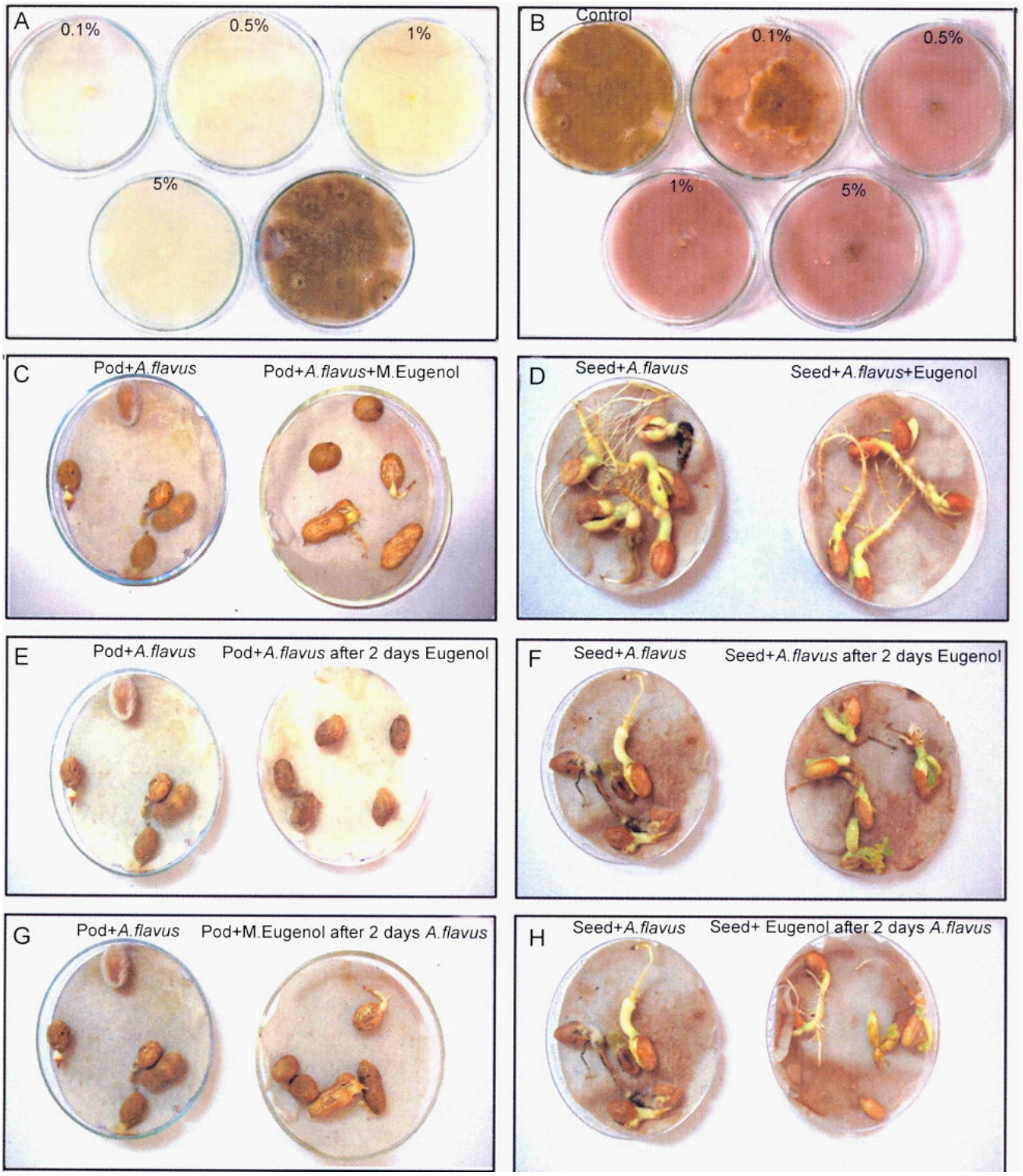

Fig. 1—(A): Inhibition of *Aspergillus flavus* colonization in potato dextrose medium with various concentrations of methyleugenol; (B): In peanut meal agar (1%) by various concentration of methyleugenol; (C): on inhibition of peanut pod by methyleugenol (0.5%) immediately after inoculation with *Aspergillus flavus*; (D): Methyleugenol (0.5%) treatment immediately after inoculation of toxic *Aspergillus flavus*; (E): In Pod by methyleugenol (0.5%) treatment before two days of inoculation with *Aspergillus flavus*; (F): In kernel by methyleugenol (0.5%) treatment before two days of inoculation with *Aspergillus flavus*; (G): In pod by methyleugenol (0.5%) treatment after two days of inoculation with *Aspergillus flavus*; and (H): In kernel by methyleugenol (0.5%) treatment after two days of inoculation with *Aspergillus flavus*.

(Table 1; Fig.1 A, B). Methyleugenol (0.1%) could prevent *A. flavus* colonization (100%) on PDA and 56% on peanut agar meal. For further experimentations on inhibition of pod and kernel infection, methyleugenol (0.5%) was used.

Inhibition of pod and kernel infection—Inoculation of *A. flavus* on peanut susceptible variety (K-134) pods and kernels showed infection (100%) after 6 days of treatment in experiment II. However, peanut pods and kernels surface treated with methyleugenol (0.5%) immediately after inoculation completely inhibited *A. flavus* colonization (Table 2) both in pods (Fig.1C) and kernel (Fig. 1D). Similarly, aflatoxin biosynthesis (AfB1) by *A. flavus* in peanut pod and kernels was inhibited by methyleugenol (0.5%), when applied immediately after inoculation (Table 2). Experiment III, methyleugenol also proved effective in complete prevention (100 %) of *A.flavus* colonization (Table 2) both in pods (Fig. 1 E) and kernels (Fig. 1 F). Similarly aflatoxin B1 was traced in pod and kernel after 6 days after incubation (Table 3). Methyleugenol showed complete inhibition (100 %) of *A. flavus* colonization when applied 2 days after inoculation (Table 2) both in pods (Fig.1G) and kernels (Fig. 1 H). AfB1 was not detected after 6 days of incubation (Table 2).

Discussion

Pathogenesis of peanut kernels by *A.flavus* and subsequent production of aflatoxin is quite complex and different from other soil borne diseases[9] Availability of limited genetic tolerant and low adaptability of available technologies for pre and post harvest prevention of aflatoxin made this problem, still unsolved in peanut. Several antifungal chemicals have been used for the preservation of stored grains[10]. Though several workers demonstrated the antifungal activity of clove, eugenol and other plant derived chemicals, no attempt has been made on inhibition of *A. flavus* colonization and aflatoxin contamination in peanut under post-harvest storage. It is also true that, large scale usage of eugenol against *A. flavus* on peanut is expensive and adoption level will be low. The present study demonstrated the usage of methyleugenol (4-allyl 1, 2 dimethoxybenzene) compound which is an ideal alternative to protect peanuts from post- harvest infection of *A.flavus* and aflatoxin contamination. Methyleugenol at 0.5 % concentration was proved to be effective, in terms of inhibition and cost effectiveness. Methyleugenol (0.5 %) completely inhibited *A.flavus* colonization and synthesis of aflatoxins, compared to 80 per cent infection in control. Prophylactic treatment of peanut pods and kernels with methyleugenol (0.5%) followed by inoculation of *A. flavus* infection showed complete prevention of *A. flavus* colonization and aflatoxin (AfB1) biosynthesis. It is suggested that methyleugenol (0.5%) can be used as spray to prevent

Table 2—Effect of Methyleugenol (0.5%) on aflatoxin B_1 biosynthesis by *A. flavus* in peanut

Methyleugenol treatment	Aflatoxin B_1 (μg/kg)		Inhibition over control (1%)	
	Pod	Seed	Pod	Seed
Control (with Methyl eugenol)	243.4	1114.3	0	0
Immediately after artificial inoculation	0	0	100	100
Two days before artificial inoculation	0	0	100	100
Two days after artificial inoculation	0	0	100	100

Table 1—Effect of methyleugenol on antifungal activity against *A.flavus* colonization on media and substrate (peanut pod and kernel)

Methyl eugenol Treatment (%)	Fungal growth (Diameter; cm)		Inhibition over control (1 %)		Methyl eugenol treatment (0.5 %)	*A.flavus* infection (%)		Inhibition over control (%)	
	PDA	PMA	PDA	PMA	Control (without eugenol)	Pod	Kernel	Pod	kernel
Control	9.0	9.0	-	-		100%	100%	0	0
0.1	0	3.9	100	56.7	Immediately after artificial inoculation	0	0	100	100
0.5	0	0	100	100	Two days before artificial inoculation	0	0	100	100
1.0	0	0	100	100	Two days after artificial inoculation	0	0	100	100
5.0	0	0	100	100					

PDA; Potato Dextrose Agar; PMA: Peanut Meal Agar

post-harvest *A. flavus* colonization and aflatoxin synthesis. Spray of methyleugenol (0.5%) directly on peanut kernels against *A. flavus* is debatable in terms of food safety.

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