INHIBITION OF AFLATOXIN PRODUCTION AND GROWTH OF *Aspergillus flavus* BY CITRONELLA OIL

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ABSTRACT

The activity of citronella oil on aflatoxin production and the growth of *Aspergillus flavus* was studied. *Aspergillus flavus* IMI 242684 was grown on PDA and maize grain for 28 days. It was found that the fungal growth on PDA was inhibited by citronella oil at 0.2% (v/v) whereas the growth, aflatoxin production and spore production in maize grain were inhibited at 1.0% or more. The growths of *A. flavus* M 113, *A. flavus* S 156 on PDA were inhibited by citronella oil at 0.4 % whereas the growth of *A. parasiticus* IMI 10256 was inhibited at 0.2%.

KEYWORDS : aflatoxin, herb, citronella oil, *Aspergillus flavus*, *Aspergillus parasiticus*, aflatoxin producing fungi

1. INTRODUCTION

Aflatoxins are the most notorious of the mycotoxins causing acute and chronic toxicoses in animals and human and produce four distinct effects: acute liver damage; liver cirrhosis; tumour induction amd teratogenesis [1,2]. Outbreaks of aflatoxicosis in farm animals have been reported from many areas of the world [1]. The species of the *Aspergillus flavus* group that produce aflatoxins include *A. flavus, A. parasiticus, A. nomius, A. tamarii* and *A. bombycis* [3-6]. These fungi can grow on a wide range of agricutural commodities. Aflatoxins can be produced in preharvest as well as in stored products [3].

Natural compounds from plants have been used traditionally to preserve foods in countries like Japan, India and Russia [7]. The extracts and powders of some local plants show the ability to suppress the growth of toxigenic fungi and hence, toxin production in synthetic media [8-10]. Plant-produced compounds are becoming of interest as a source of safer and more effective substances than synthetically produced antimicrobial agents. Many essential oils have also been reported as effective inhibitors of fungal growth and aflatoxin production [11]. Oils obtained from higher plant parts have been shown to contain antitoxigenic properties [12,13]. The present study was undertaken in order to observe the effect of various concentrations of citronella oil (*Cymbopogon winterianus* Jewitt) on the growth of *A. flavus* and aflatoxin production when the fungi was grown on PDA and maize grain.

2. MATERIALS AND METHODS

Fungal strains

Aspergillus flavus IMI 242684 and *A. parasiticus* IMI 102566 were purchased from the International Mycological Institute, England. *Aspergillus flavus* M 113 and *A. flavus* S 156 were obtained from the Division of Plant Pathogen and Microbiology, Ministry of Agriculture, Thailand.

Cultural conditions

Cultivation on PDA. Appropriate amounts of citronella oil were added into sterile PDA to obtain the concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 %. PDA without citronella oil served as control. *Aspergillus flavus* IMI 242684, *A. flavus* M 113, *A. flavus* S 156 and *A. parasiticus* IMI 102566 were cultured on PDA in petri dishes as single colonies and incubated at room temperature for 28 days. Diameters of fungal colonies were measured every 7 days.
Cultivation in maize grain. Sterile maize grains (50g) with initial moisture content of 22-23% [14] were mixed with appropriate amounts of citronella oil (1%, 2%, 3%, 4% and 5%). The sample without citronella oil was used as control. The samples were then inoculated with spores of *A. flavus* IMI 242684 (10^6 spores/g) and incubated at room temperature for 28 days. Samples were taken for analysis at day 3, 5, 7, 14, 21 and 28.

Fungal growth observation

Fungal growth of *A. flavus* IMI 242684 in maize grain was visually assessed using a semiquantitative scale, viz. (0) no growth; (1) very little growth; (2) 25% of the grains covered; (3) 50% of the grains covered; (4) 75% of the grains covered; (5) all of the grains covered[15].

Spore count

Spores of *A. flavus* IMI 242684 were harvested from maize grain using sterile distilled water plus 0.1% tween 80. The spore suspension was filtered and pooled in a sterile bottle and the numbers of spores were counted using a haemacytometer.

Aflatoxin analysis

Aflatoxin extraction from the samples (50g) was according to the modified methods of Seitz and Mohr [16] and Lichrolut Vacuum Manifold. Aflatoxins were analyzed by HPLC (Shimadzu) using the the following condition: flow rate of 1ml/min, UV spectrophotometric detector at 365 nm, reverse phase column C18 and mobile phase solvent of methanol-water-acetic acid (30:63:7). The amount of aflatoxins was calculated from the chromatogram by comparison to the standard aflatoxin chromatogram.

Statistical analysis

All experiments were repeated at least four times. The experimental data were analyzed statistically by the analyses of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) at the 5% level.

3. RESULTS AND DISCUSSION

The effects of various concentrations of citronella oil on the growth of aflatoxin producing fungi on PDA are given in Tables 1-4. The results showed that the fungal growths were inhibited at concentrations of 0.2-1.0%. The citronella oil at concentration of 0.2% could inhibit the growth of *A. flavus* IMI 242684, *A. flavus* M 113, *A. flavus* S 156 and *A. parasiticus* IMI 102566 for 21, 7, 7 and 21 days, respectively whereas all fungi were completely inhibited for 28 days at concentration of 1.0%. Statistical analyses on the effects of citronella oil on the growth of aflatoxin producing fungi are given in Table 5. The growth of all aflatoxin producing fungi were significantly reduced at concentrations of 0.2% or more.

When *A. flavus* IMI 242684 was grown in maize grain with various concentrations of citronella oil (1-5%), the results showed that the growth and aflatoxin formation were inhibited for only 3 days at concentration of 1% whereas fungal growth and aflatoxin production were completely inhibited for 28 days at concentration of 5% (Tables 6,7 and Fig. 1). Statistical analyses on the effects of citronella oil on the growth of *A. flavus* IMI 242684 and aflatoxin production are given in Tables 8 and 9. The growth of *A. flavus* was significantly inhibited at concentrations of 2% or more whereas the aflatoxin production was significantly reduced at concentration of 1%.

Table 1. Colonies' diameter (cm) of *A. flavus* IMI 242684 on PDA with various concentrations of citronella oil.

Incubation Period	Concentration (%)					
(days)	Control	0.2	0.4	0.6	0.8	1.0
7	9.00	0.00	0.00	0.00	0.00	0.00
14	9.00	0.00	0.00	0.00	0.00	0.00
21	9.00	0.00	0.00	0.00	0.00	0.00
28	9.00	2.06	0.00	0.00	0.00	0.00

Table 2. Colonies' diameter (cm) of *A. flavus* M 113 on PDA with various concentrations of citronella oil.

Incubation Period	Concentration (%)					
(days)	Control	0.2	0.4	0.6	0.8	1.0
7	6.39	0.00	0.00	0.00	0.00	0.00
14	8.97	1.87	0.62	0.57	0.00	0.00
21	9.00	5.05	2.64	2.57	0.13	0.00
28	9.00	7.22	4.81	4.23	0.58	0.00

Table 3. Colonies' diameter (cm) of *A. flavus* S 156 on PDA with various concentrations of citronella oil.

Incubation Period	Concentration (%)					
(days)	Control	0.2	0.4	0.6	0.8	1.0
7	8.37	0.00	0.00	0.00	0.00	0.00
14	8.89	0.28	0.00	0.00	0.00	0.00
21	9.00	1.78	0.00	0.00	0.00	0.00
28	9.00	2.91	0.00	0.00	0.00	0.00

Table 4. Colonies' diameter (cm) of *A. parasiticus* IMI 102566 on PDA with various concentrations of citronella oil.

Incubation Period (days)	Concentration (%)					
	Control	0.2	0.4	0.6	0.8	1.0
7	9.00	0.00	0.00	0.00	0.00	0.00
14	9.00	0.00	0.00	0.00	0.00	0.00
21	9.00	0.00	0.00	0.00	0.00	0.00
28	9.00	6.18	0.00	0.00	0.00	0.00

Table 5. Analysis of difference for the effect of citronella oil on the growth of aflatoxin producing fungi on PDA.

Concentration (%)	Mean of colonies' diameter (cm)*			
	*A. flavus*IMI 242685	*A. flavus*M 113	*A.. flavus* S 156	*A. parasiticus* IMI 102566
Control	9.00 [a]	8.34 [a]	8.82 [a]	9.00 [a]
0.2	0.52 [b]	3.53 [b]	1.25 [b]	1.55 [b]
0.4	0.00 [b]	2.02 [b c]	0.00 [c]	0.00 [b]
0.6	0.00 [b]	1.84 [c]	0.00 [c]	0.00 [b]
0.8	0.00 [b]	0.18 [c]	0.00 [c]	0.00 [b]
1.0	0.00 [b]	0.00 [c]	0.00 [c]	0.00 [b]

* Means in the same column with different superscripts are significantly different($P<0.05$)

Table 6. Effect of various concentrations of citronella oil on the growth of *A. flavus* IMI 242684 in maize grain.

Incubation Period (days)	Concentration (%)					
	Control	1	2	3	4	5
3	3	0	0	0	0	0
5	4	1	0	0	0	0
7	5	1	0	0	0	0
14	5	3	1	1	0	0
21	5	5	4	2	1	0
28	5	5	5	3	2	0

Note 0 = no growth
1 = very little growth
2 = 25% of the grains covered with mycelium
3 = 50% of the grains covered with mycelium
4 = 75% of the grains covered with mycelium
5 = all of the grains covered with mycelium

Table 7. Numbers of spores of *A. flavus* IMI 242684 in maize grain (spores/g) with various concentrations of citronella oil.

Incubation Period (days)	Concentration (%)					
	Control	1	2	3	4	5
3	6.50×10^{8}	0.00	0.00	0.00	0.00	0.00
5	8.60×10^{8}	6.00×10^{7}	0.00	0.00	0.00	0.00
7	1.40×10^{9}	1.70×10^{8}	0.00	0.00	0.00	0.00
14	2.40×10^{9}	7.60×10^{8}	1.42×10^{8}	2.60×10^{7}	0.00	0.00
21	3.02×10^{9}	1.48×10^{9}	2.32×10^{8}	2.12×10^{8}	2.60×10^{7}	0.00
28	5.98×10^{9}	2.89×10^{9}	1.95×10^{9}	3.84×10^{8}	1.64×10^{8}	0.00

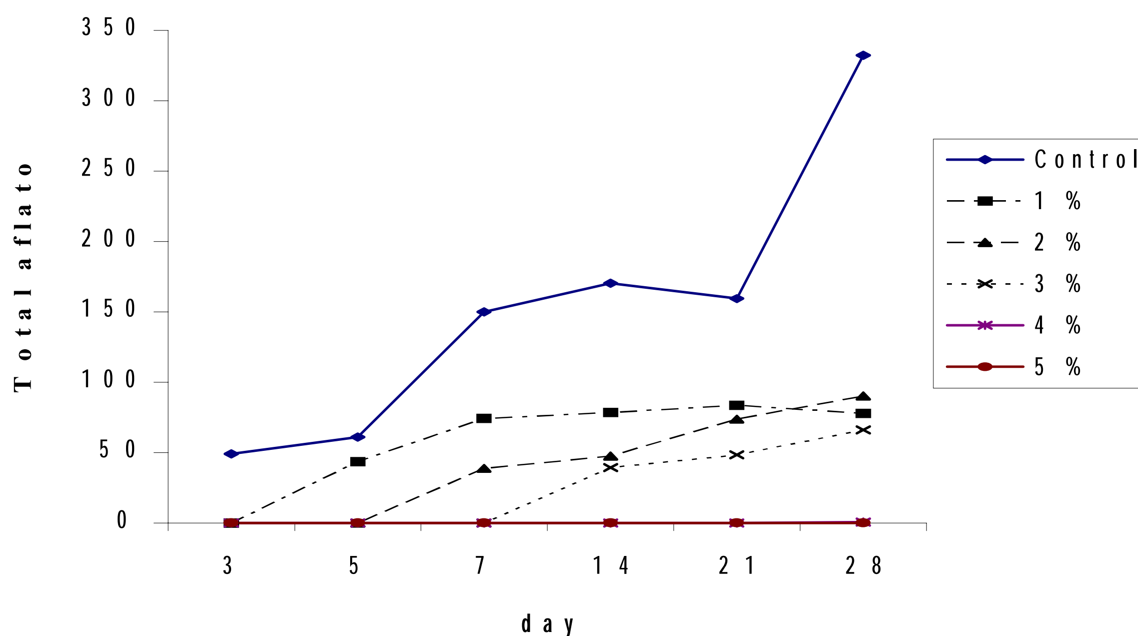

Fig. 1 Total aflatoxin production (B1&B2) of *A. flavus* IMI 242684 in maize grain at various concentrations of citronella oil.

Table 8. Analysis of difference for the effect of citronella oil on the growth of *A.flavus* IMI 242684 in maize grain.

Concentration (%)	Average of spores* (spores/g)
Control	$2.3850 \times 10^{9\,a}$
1	$8.8833 \times 10^{8\,b}$
2	$3.8775 \times 10^{8\,c}$
3	$1.0367 \times 10^{8\,c}$
4	$0.3167 \times 10^{8\,c}$
5	0.0000^{c}

* Means in the same column with different superscripts are significantly different (P<0.05)

Table 9. Analysis of difference for the effect of citronella oil on the production of aflatoxin of *A. flavus* IMI 242684 in maize grain.

Concentration (%)	Average of total aflatoxins* (µg/g)
Control	153.79^{a}
1	59.69^{b}
2	41.79^{b}
3	25.67^{b}
4	0.11^{b}
5	0.00^{b}

* Means in the same column with different superscripts are significantly different (P<0.05)

More than 280 plant species have been investigated for their inhibitory effect on toxigenic *Aspergillus* spp. and nearly one hundred of them had some activity on growth or toxin production by these fungi [17]. Antifungal and anti-toxigenic activities of essential oils have also been investigated by many researchers. Antimicrobial activity of essential oils depends not only on their components but also on the chemical structure of these components [18,19].

Mahmoud [20] reported that aqueous extracts of *Lupinus albus* (Leguminosae), *Ammi visnaga* (Umbelliferae) and *Xanthium pungens* (Compositae) at five different concentrations (0.2%, 0.4%, 0.6%, 0.8% and 1.0%) inhibited mycelial growth and aflatoxin formation in a chemically defined medium. The extracts of these plants inhibited aflatoxin production by inhibiting the growth of *A. flavus*. The nature of the plant extract also affected the ratio of B1 to B2, and there was no correlation between the inhibition of aflatoxins or growth of the fungus and the resultant B1: B2 ratio.

Five oils, namely geraniol, nerol and citronellol (aliphatic oils), cinnamaldehyde (aromatic aldehyde) and thymol (phenolic ketone), completely suppressed growth and aflatoxin synthesis [11]. The minimum inhibitory concentration (MIC) of these oils revealed that geraniol, nerol and citronellol were effective at 500 ppm (0.05%) while thymol and cinnamaldehyde were highly effective at doses as low as 250 and 200 ppm (0.025 and 0.02%), respectively. Some of these essential oils could prevent fungal growth and toxin formation for up to 8 days. After 15 days of incubation, it was found that toxin production was greater than controls. Some constituents in each oil play an important role in its antifungal action [21-23].

Misra *et al.* [24] tested the antifungal activities of essential oil of *Cymbopogon martinii* (lemongrass) on *A. flavus, A. fumigatus* and *A. parasiticus*. It was found that the active component was geraniol.

Sinha *et al.* [25] studied the effect of clove and cinnamon oils on growth and aflatoxin production by *A. flavus* in SMKY liquid medium and maize. Significant reduction ($P < 0.05$) in the elaboration of aflatoxin in liquid culture after treatment with more than 100 µg/ml (0.01%) of these compounds was recorded whereas cinnamon oil exhibited maximum inhibitory action and reduced 78% aflatoxin formation on maize at 1000 mg/kg (0.1%) when *A. flavus* was cultured for 10 days.

Our results showed that citronella oil at certain concentrations could inhibit both growth and aflatoxion produciton by all fungi tested. However, the dosage for controlling aflatoxin producing fungi in maize grain was much higher than on PDA . This finding is similar to the report of Montes-Belmont and Carvajal [17] who tested the effects of 11 plant essential oils for maize kernel protection against *A. flavus*. The results showed that essential oils of *Cinnamomum zeylanicum* (cinnamon), *Mentha piperita* (peppermint), *Ocimum basilicum* (basil), *Origanum vulgare* (origanum), *Teloxys ambrosioides* (the flavoring herb *epazote*), *Syzygium aromaticum* (clove), and *Thymus vulgaris* (thyme) caused a total inhibition of fungal development on maize kernel at the optimal dosage between 3-8%. Davidson and Parish [26] explained that in a food system such as maize grain, there were several factors that could affect the effectiveness of the compounds: pH environment, lipids that decreased activity of hydrophobic compounds, and proteins that might cause binding of some compounds and reduce activity. When dealing with grain protection, fumigation is the preferred method for applying substances into the bulks in order to control the biotic factors which damage the grain [27].

4. CONCLUSION

Antifungal chemicals have been used for the preservation of stored grains [27]. Due to health and economic considerations, natural plant extracts may provide an alternative method to protect food and feed from fungal contamination [28]. Citronella oil can be effective inhibitor of fungal growth and aflatoxin production if used in sufficient amount, however, the optimal dosage was too high for practical application.

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