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Fungicidal and anti-aflatoxigenic effects of the essential oil of *Cymbopogon citratus* (DC.) Stapf. (lemongrass) against *Aspergillus flavus* Link. isolated from stored rice

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ABSTRACT

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Aims: To develop a natural fungicide against aflatoxigenic fungi, to protect stored rice, using the essential oil of lemongrass.

Methods and Results: *Aspergillus flavus* Link. was isolated from stored rice and identified as an aflatoxigenic strain. Lemongrass oil was tested against *A. flavus* and the test oil was fungistatic and fungicidal against the test pathogen at 0·6 and 1·0 mg ml^{-1}, respectively. Aflatoxin production was completely inhibited at 0·1 mg ml^{-1}. The results obtained from the thin layer chromatographic bioassay and gas chromatography indicated citral a and b as the fungicidal constituents in lemongrass oil. During the fumigant toxicity assay of lemongrass oil, the sporulation and the mycelial growth of the test pathogen were inhibited at the concentrations of 2·80 and 3·46 mg ml^{-1}, respectively.

Conclusion: Lemongrass oil could be used to manage aflatoxin formation and fungal growth of *A. flavus* in stored rice.

Significance and Impact of the Study: Currently, fungicides are not used to control fungal pests or mycotoxin production on stored rice. Rice treated with the essential oil of lemongrass could be used to manage fungal pests as well as the insect pests in stored rice. The essential oil is chemically safe and acceptable to consumers, as synthetic chemical fungicides can cause adverse health effects to consumers.

Keywords: aflatoxin, *Aspergillus flavus*, essential oils, stored rice.

INTRODUCTION

Rice is the staple food among Sri Lankans. However, specific conditions of temperature, relative humidity and moisture content of rice, which arise during storage, may contribute to the rapid deterioration of stored rice by promoting fungal growth (Christensen and Saucer 1992). Fungal infections may discolour grains, change its chemical and nutritional characteristics, reduce germination and most importantly, contaminate it with mycotoxins, such as aflatoxins which are highly toxic to man and animals (Paster *et al.* 1993). *Aspergillus flavus* Link. is one of the major storage fungi found regularly in important cereals cultivated in the world (Paster 1995), which produces aflatoxins such as aflatoxin B_1, B_2, G_1, G_2 (http://vm.cfsan.fda.gov/~mow/chap41.html). Breckenridge *et al.* (1986) reported that aflatoxin B_1 (AFB_1) was detected in rice, and 96% of the parboiled rice samples tested contained AFB_1. Though the aflatoxin content in these samples were below the UNICEF/WHO/FAO permissible levels, the contamination is very critical even at low levels, as rice is consumed in significant quantities. As rice is a good substrate for aflatoxin B_1 production, the importance of research work in controlling the growth of *A. flavus* and aflatoxin contamination of the stored rice was investigated (Ilago and Juliano 1982).

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Protection of stored grain currently relies on synthetic pesticides such as the fumigant phosphine or dust formulations such as pirimiphos methyl (Anonymous 1995). However, the widespread use of these pesticides have significant drawbacks including increased cost, handling hazards, concern about pesticide residues on grains, and threat to human health and environment (Paster and Bullerman 1988). Public awareness of these risks has increased interest in finding safer insecticides or alternative stored product protectants to replace synthetic chemical pesticides. One such alternative is the use of natural plant protectants that have pesticidal activity, because they tend to have low mammalian toxicity, less environmental effects and wide public acceptance (Liu and Ho 1999; Hamilton-Kemp *et al.* 2000; Singh and Tripathi 1999; Paster *et al.* 1995; Don-Pedro 1996).

Many plant derivatives have shown to be effective against stored-product pests (Huang *et al.* 2000; Liu and Ho 1999; Huang *et al.* 1997). Essential oils are plant volatiles containing monoterpenes, sesquiterpenes and phenyl propionoids. Essential oils of *Cymbopogon citratus*, *C. nardus, Cinnamomum zeylanicum* and *Alpinia calcarata* induced 100 % mortality and caused significant reduction in progeny production by rice weevil *Sitophilus oryzae* and grain moth *Sitotroga cerealella* (Paranagama *et al.* 2001a, 2002a, b). Fungicidal constituents from essential oils have been identified against various fungi (Batt *et al.* 1983; Adegoke *et al.* 2000; Dorman and Deans 2000; Ranasingha *et al.* 2002). Different extracts of cinnamon and black pepper have been previously reported to inhibit the mycotoxin formation even without inhibiting the mycelial growth (Paster 1994). Inhibition of *A. flavus* and aflatoxin formation by the essential oil of *Cinnamomum zeylanicum* and *C. nardus* has already been reported (Jayaratne *et al.* 2002).

Cymbopogon citratus (DC.) Stapf. (Family Poaceae), a spice plant grown in Sri Lanka, commonly known as lemongrass, is of West Indian origin and yields an essential oil with high content of citral (>70%) (Paranagama 1991). Citral is an oxygenated terpenoid (aldehyde), which has been identified as a compound exhibiting antifungal properties (Batt *et al.* 1983).

The objective of the current study was to investigate the fungicidal and the anti-aflatoxigenic effects of the essential oil of lemongrass against *A. flavus* with the aim of developing a cost effective and environmental friendly treatment system to control pests on stored rice/paddy.

MATERIALS AND METHODS

The essential oil of *C. citratus* was of commercial origin and purchased from EOAS Organics Pvt. Ltd., Rathmalana, Sri Lanka. Aflatoxin B_1 (AFB_1) was purchased from Supelco Co., Bellefonte, PA, USA.

Three samples of rice were collected from Kurunegala district in Sri Lanka. Using each sample, 50 seeds were placed on moist blotting paper, according to the method described in Geeta and Reedy (1990). After 7 days *A. flavus* was isolated from rice samples and maintained on potato dextrose agar (PDA, 20 g potatoes, 20 g glucose, 20 g agar in 1 l distilled water). Identification of *A. flavus* isolate was confirmed by the International Mycological Institute (IMI), United Kingdom.

To determine fungitoxicity of lemongrass oil, 50 ml of SMKY semi-synthetic liquid medium (20 g sucrose, 7 g yeast extract, 3 g KNO_3, 0·5 g $MgSO_4{\cdot}7H_2O$ in 1 l water) was dispensed into 100 ml Erlenmeyer flasks and autoclaved at 121°C and 1·03 kg cm^{-2} for 15 min (Sterilemax, Series 1118, Barnstead Thermolyne, Dubuque, IA, USA). Different doses of the oil (5–50 mg) and 5 μl of the surfactant (25% Tween®-20 solution in sterile water) were added and mixed with the culture media separately. A fungal disc of 5 mm diameter from the periphery of a 7-day-old *A. flavus* culture, grown on PDA, was incorporated into each flask. The cultures were placed on an orbital shaker at $0{\cdot}5 \times 100$ rev min^{-1} (Lab-line, Melrose Park, IL, USA) at ambient temperature (28 ± 2°C) for 7 days. Samples without any oil treatment were considered as controls. To determine the mycelial dry weight, the cultures were filtered under vacuum through a preweighed Whatman No. 1 filter paper at the end of the incubation period. The paper containing the mycelium was washed twice with 10 ml distilled water and dried in an oven (Gallenkamp, Loughborough, Leices, UK) at 110°C to a constant weight. Experimental design was a complete randomized design (CRD) with five replicates. The minimum inhibitory concentration (MIC or the fungistatic concentration) of the test oil was taken as the lowest concentration, which inhibited the growth of the test fungus in the liquid medium (fungus however revived within 7 days on untreated PDA). The lowest concentration of the oil, which killed the test fungus, was considered as the minimum lethal concentration (MLC or the fungicidal concentration) (no revival was observed on fresh PDA).

To analyse aflatoxin B_1 in liquid cultures, an aliquot (20 ml) from the above liquid culture filtrate (after separating mycelial mats, if any) was extracted twice with 15 ml portions of $CHCl_3$. The combined $CHCl_3$ extracts were dried on anhydrous Na_2SO_4 and solvent was evaporated to dryness on a rotary evaporator (R-114 & B-480, Büchi Labortechnik AG, Flawil, Switzerland). Each residue was redissolved in 1 ml $CHCl_3$ and spotted (10 μL) on a precoated silica gel G TLC plate (0·5 mm thickness, 5 × 10 cm, Merk, Germany), separately. Aflatoxin B_1 ($3{\cdot}25$ μg ml^{-1}) standard was also spotted along with the extracts and all TLC plates were developed in the solvent system $CHCl_3$: methanol : water (88·5 : 11·0 : 0·5). Subsequently, the spots on TLC were

viewed under u.v. light (365 nm). The presence of AFB_1, AFB_2, AFG_1 and AFG_2 were confirmed by spraying 25% H_2SO_4 in water (Nesheim and Brumley 1981).

The residues which showed the presence of aflatoxins were further purified using a silica gel G-60 mini column (15 cm height, 0·5 cm internal diameter) and 100 μl of $CHCl_3$. The column was washed successively three times with 1·5 ml of (i) *n*-hexane, (ii) diethyl ether (iii) $CHCl_3$: methanol (97 : 3). The last three fractions were collected, combined and evaporated at 40°C using a rotary evaporator. The residues were saved for high performance liquid chromatography (HPLC).

Aflatoxin residues were derivatized with trifluoro acetic acid (TFA) according to the method described by Dayananda (1990). The derivatised aflatoxin samples and AFB1 were analysed by reverse phase (C-18 column) HPLC system (1100 series, Agilent Technologies Deutschland GmbH, Waldbronn, Germany) with a fluorescence detector. The solvent system was water : methanol : acetonitrile (60 : 20 : 20) with 360 nm and 445 nm excitation and emission filters. The flow rate was at 1 ml min^{-1}. Three replicates were injected (10 μl) and the quantification of AFB_1 in samples was performed using the peak height.

To evaluate the fumigant effect of lemongrass oil, glass screw cap vials (15 ml), containing 1·5 ml of PDA, were autoclaved and the vials were inoculated with 100 μl of spore suspension (10^6 conidia ml^{-1}) of *A. flavus* obtained from 14 days old PDA slants. On the underside of the screw caps of the vials, cellulose sponges (1 cm diameter, Scotchbrite, USA) were placed aseptically and different doses of the test oil were applied on to the sponges separately. The screw caps were replaced and incubated at ambient temperature for 10 days (Goubran and Holmes 1997). The experimental design was CRD with five replicates. Samples without oil served as the controls.

After the incubation period, the concentrations at which the sporulation and mycelial growth inhibited were recorded. Whenever sporulation was evident, 10 ml of water and 5 μl of 25% Tween®-20 were incorporated into the vial and shook vigorously to dislodge the conidia. Then the conidial density was determined using a neubaeur counter (Hauser Scientific, USA).

To determine the antifungal constituents of lemongrass oil, the oil was streaked along the horizontal axis of a TLC plate (20 × 20 cm) as a band and developed in chloroform : toluene (3 : 1, v/v) solvent system. The developed plate was air dried and cut into three parts (a, b and c). Part 'a' was sealed in a polythene bag and stored at 4°C for gas chromatographic (GC) analysis. Part 'b' was sprayed with 25% H_2SO_4 (v/v) and heated at 110°C to visualize the separated constituents. Part 'c' was sprayed with a spore suspension of *A. flavus* in Czepax dox nutrient medium and incubated at ambient temperature in a moist chamber for 7 days (Müller-Reibau *et al.* 1995).

The zones on 'a' which are comparative to the inhibitory zones on 'c' were scraped and dissolved in 3 ml of dichloromethane and filtered sample was concentrated under a dry nitrogen stream, to 1 ml. The essential oil and the extract from the TLC were analysed on GC (5890 series III; Hewlett Packard, Palo Alto, CA, USA) equipped with a Carbowax 30 m capillary column (J & W Scientific, Folsom, CA, USA) and flame ionization detector. The GC conditions used were carrier gas, Helium (1·5 ml min^{-1}), initial temperature 40°C and then heated to 210°C at a rate of 5°C min^{-1} and kept at 210°C for 10 min. The chemical and fungicidal constituents were identified by using standard compounds and previously published data (Paranagama 1991).

The mycelial dry weights and AFB_1 concentrations at each oil concentration were statistically analysed by one-way ANOVA using Minitab (State College, PA, USA) statistical package. Comparisons of mycelial dry weights and AFB_1 concentrations at each treatment were made using Tukey's pair-wise comparison test at 0·05% probability level.

RESULTS

Aspergillus flavus [IMI, U.K. (IMI # 384871)] was responsible for the production of aflatoxin in stored rice. The frequencies of occurrence of *A. flavus* in the parboiled and two white raw rice samples were 83·5, 15·3, and 2·4%, respectively, indicating the higher frequency of *A. flavus* in parboiled rice.

Lemongrass essential oil was fungistatic and fungicidal against *A. flavus* at 0·60 and 1·0 mg ml^{-1} concentrations in liquid culture media, respectively. The mycelial dry weights at the concentrations of 0·1, 0·2 and 0·3 mg ml^{-1} oil were not significantly different from that of the control ($P > 0·05$). Thin layer chromatography analysis of aflatoxin extracts showed fluorescing spots under long wave u.v with a blue fluorescing spot parallel to AFB_1 standard. When sprayed with H_2SO_4, spots of green and blue turned to yellow and green colour under u.v., confirming the presence of aflatoxins. Aflatoxins were not detected in the liquid culture at concentrations higher than 0·2 mg ml^{-1}, though mycelial growth was yet evident. The results revealed that at 0·1 mg ml^{-1} lemongrass oil, the AFB_1 concentration was 526 ppb. There was a significant difference in aflatoxin production of control compared with the oil treated samples (Table 1).

The results obtained for the fumigant effect bioassay revealed that the sporulation of *A. flavus* was completely inhibited at 2·8 mg ml^{-1} test oil concentration (Fig. 1). Mycelial growth because of the fumigant effect was not observed above the oil concentration of 3·5 mg ml^{-1}.

Table 1 Influence of the essential oil of lemongrass on the mycelial growth of *A. flavus*

Concentration of oil (mg ml^{-1})	Mycelial dry weight (g)*	AFB_1 (ppb)†
0·1	0·364 ± 0·001[a]	526 ± 32[a]
0·2	0·408 ± 0·009[a]	n.d.
0·3	0·367 ± 0·008[a]	n.d.
0·4	0·189 ± 0·007[b]	n.d.
0·5	0·046 ± 0·001[c]	n.d.
Control	0·422 ± 0·027[a]	2070 ± 70[b]

*Mean of five replicates. †n = 3. n.d., not detected. *†Mean followed by similar letters in a column are not significantly different ($P > 0·05$).

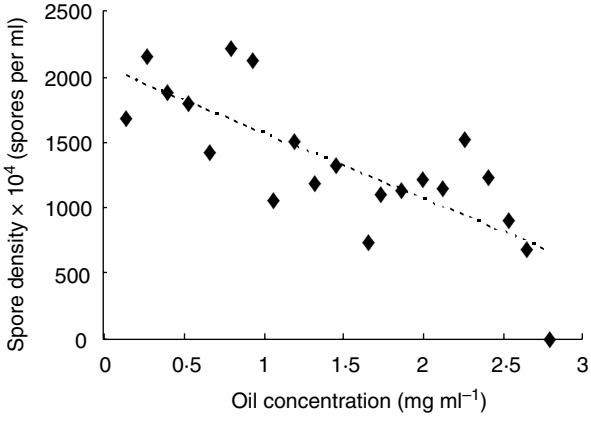

Fig. 1 The fumigant effect of the essential oil of *C. citratus* on the sporulation of *A. flavus*. ($n = 5$)

Analysis of lemongrass oil by TLC confirmed the identity of citral a & b as the fungicidal constituents in lemongrass essential oil.

DISCUSSION

The main pathogen isolated from all Sri Lankan rice samples was *A. flavus*. Breckenridge *et al.* (1986) has previously reported that *A. flavus* is highly abundant in parboiled rice samples. The inhibition of the myclelial growth of *A. flavus*, isolated from rice, was previously reported when treated with essential oils of *C. zeylanicum* and *C. nardus* with the minimum lethal concentrations of 1 and 4 mg ml^{-1}, respectively (Jayaratne *et al.* 2002). Many reports are available on the effect of the essential oils on the growth and the aflatoxin formation of *A. flavus* (Sharma *et al.* 1979; Adegoke *et al.* 2000). Baratta *et al.* (1998) has reported 91% inhibition of the growth of *A. niger* in liquid culture media, when treated with 1 μl ml^{-1} lemongrass oil. A study carried out by Batt *et al.* (1983) has revealed the complete inhibition of the mycelial growth and the aflatoxin production of *A. parasiticus* at 3·3 mg ml^{-1} of citral. Gueldner *et al.* (1985) have tested several organic compounds and the results revealed that aldehydes were fungitoxic against *A. flavus*. This is in agreement with the present study as citral a & b were identified as the fungicidal compounds.

The effects of different extracts and powdered spices of cinnamon and pepper on *A. flavus* have been studied and the results revealed that the test samples inhibited the production of aflatoxin without inhibiting the mycelial growth (Paster 1994 and Bullerman 1974). The essential oils of *C. zeylanicum* and *C. nardus* inhibited the production of aflatoxin at or above the concentrations of 0·4 and 0·6 mg ml^{-1}, respectively (Jayaratne *et al.* 2002). The results of the present study revealed that the lemongrass oil has a similar effect on the production of aflatoxins.

When applying a pesticide to grain in storage, the most effective method is fumigation. Paster *et al.* (1995) have highlighted the importance of fumigation in order to control the biotic factors, which damage the stored grain. Thus the findings in the fumigant activity test is more important than the MIC value, though higher concentration of lemongrass oil was required to inhibit the mycelial growth of *A. flavus*. It was reported that lemongrass oil has not demonstrated fungicidal activity against *Penicillium digitatum* and *P. italicum* when applied as a fumigant (Goubran and Holmes 1997). The essential oils of oregano and thyme, which contain phenolic compounds, have inhibited the mycelial growth and sporulation of *A. flavus* at concentrations <3 μl l^{-1} (Paster *et al.* 1995).

The use of spice plant materials is feasible in protecting rice as they are used as flavouring agents. The fungicidal efficacy and the aflatoxin suppressing property of the essential oil of *C. citratus* is evident from the current study. Therefore, the use of lemongrass oil could be recommended with further investigations on field trials, organoleptic properties and cooking quality.

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