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Inhibition of *Aspergillus flavus* Link and Aflatoxin Formation by Essential Oils of *Cinnamomum zeylanicum* (L.) and *Cymbopogon nardus* Rendle

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ABSTRACT. *The essential oils of Cinnamomum zeylanicum (L.) leaf and Cymbopogon nardus Rendle were tested for their fungistatic, fungicidal and aflatoxin suppressing efficacy against an isolate of Aspergillus flavus Link, isolated from rice. The mycelial growth and the aflatoxin formation in SMKY semi synthetic liquid medium in the presence of different concentrations of two test oils were monitored.*

At concentrations varying from 100 to 500 ppm of the essential oil of C. zeylanicum, the mycelial dry weight decreased from 0.49 to 0.01 g/50 ml. The percentage inhibition of the mycelial growth of A. flavus increased from 50 to 95 when treated with 1000 to 1800 ppm of C. nardus oil. The minimum lethal concentrations (MLCs) for the essential oils of C. zeylanicum leaf and C. nardus were 1000 and 4000 ppm, respectively. Aflatoxins were not present in the medium at or above the concentrations of 400 and 600 ppm C. zeylanicum leaf and C. nardus oils, respectively.

INTRODUCTION

Fungi on stored grain can cause extensive damage, resulting in the reduction of quantity and quality. In addition, many fungal species can produce mycotoxins, which are highly toxic to animals and humans (Paster *et al.*, 1995). Formation of mycotoxins is linked to the fungal growth. Therefore controlling factors affecting the growth prevent toxin production (Paster *et al.*, 1988).

Aflatoxins are mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* on many agricultural commodities (Grybauskas *et al.*, 1987). Rice can serve as a good substrate for aflatoxin B_1 (AFB_1) production (Usha *et al.*, 1993). Ammoniation and the use of volatile organic acids are some of the chemical methods used in the detoxification of aflatoxins. However, these chemicals cannot be incorporated to food substrates due to their hazardous nature (Farag *et al.*, 1989). A worldwide trend exists towards limiting the use of chemical fungicides in grain and food. Natural plant extracts (Paster *et al.*, 1995) and volatile products of plant origin (Hamilton-Kemp *et al.*, 2000) may provide an alternative to synthetic chemicals as they contain antifungal compounds (Singh and Tripathi, 1999).

Cinnamomum zeylanicum (L.) (cinnamon) is a spice plant grown in Sri Lanka and its stem, bark and leaves are distilled to obtain essential oils (Wijayaratne and Peiris, 1975). The major constituent of leaf oil is eugenol (77%) (Paranagama, 1991) which has previously inhibited mycelial growth and aflatoxin production of *A. parasiticus* (Farag *et al.*, 1989; Tiwari *et al.*, 1983). Buillerman (1974) has reported that the addition of ground cinnamon to the growth medium has completely inhibited aflatoxin formation by *A. flavus*.

Cymbopogon nardus Rendle (citronella) is used in cosmetic industry and the major components in the oil are borneol (5.9%), camphene (8.8%), citronellal (3.8%),

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geraniol (18.7%) and limonene (8.8%) (Paranagama, 1991). Dube *et al.* (1995) has reported the antifungal activity of *C. nardus* against *A. niger*, *A. flavus* and *A. fumigatus*. The minimum inhibitory concentration (MIC) of limonene against *A. flavus* has been reported to be 312 µg/ml (Dube *et al.*, 1995).

The objective of this study was to identify the efficacy of two essential oils obtained from *C. zeylanicum* leaf and *C. nardus*, on the growth and the aflatoxin formation by *A. flavus* isolated from paddy and rice.

MATERIALS AND METHODS

Isolation of fungi

Three samples each of rice and paddy were collected from the Kurunegala district in Sri Lanka. Using each sample, 50 seeds were placed on moist blotting paper as described by Geeta and Reddy (1990). After 7 days, the frequency of occurrence of aflatoxigenic, saprophytic and other pathogenic fungi were noted. *A. flavus* isolated from rice and paddy samples was 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.

Essential oils and aflatoxin standard

The essential oils of *C. zeylanicum* leaf and *C. nardus* were of commercial origin and were purchased from Industrial Technology Institute, Sri Lanka and Hendrik and Sons, Colombo, Sri Lanka, respectively. Aflatoxin B_1 (AFB_1) standard was purchased from Supelco, USA.

Fungitoxic properties

Fifty ml of SMKY semi synthetic liquid medium (20 g sucrose, 7 g yeast extract, 3 g KNO_3, 0.5 g $MgSO_4$, 7 H_2O in 1 l water) was incorporated into 100 ml Erlenmeyer flasks and autoclaved at 121°C for 15 min. The appropriate amounts of the oils were added into the culture media along with a surfactant (5µl of 25% Tween 20 solution in sterile water) to obtain 100 to 1000 ppm *C. zeylanicum* and 100 to 4000 ppm of *C. nardus* oil concentrations. Mycelial discs of 5 mm diameter from the periphery of a 7-day-old *A. flavus* culture grown on Potato Dextrose Agar (PDA) was incorporated into each flask. The cultures were incubated at the ambient temperature (28±2°C) for 7 days on an orbital shaker (Lab-line, USA). Samples without any oil treatment were considered as controls. The experimental design was a Complete Randomized Design (CRD) with 5 replicates.

Determination of mycelial dry weight

At the end of the incubation period, the cultures were filtered under vacuum through a preweighed Whatman No. 1 filter paper. The paper containing the mycelium was washed twice with 10 ml distilled water and dried at 110°C for 48 h and reweighed. Subsequently, the percentage inhibition of the mycelial growth was calculated as described by Singh and Tripathi (1999) using the following:

$$\textit{Percentage inhibition} = \frac{\textit{Dry weight of control} - \textit{Dry weight of treatment}}{\textit{Dry weight of control}} \times 100$$

Determination of minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC)

The minimum inhibitory concentration (MIC) of each 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) (no revival was observed on fresh PDA).

Analysis of aflatoxin B_1 in liquid cultures

An aliquot (20 ml) from the 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 rotavapor (Buchi R-114 and B-480, Switzerland). Aflatoxins were purified using a silica gel G-60 mini column (15 cm height, 0.5 cm internal diameter). The relevant extracts were introduced into the column using 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 thin layer chromatography and high performance liquid chromatography.

Thin layer chromatography (TLC)

Residues were re-dissolved in 1 ml $CHCl_3$ and spotted (10 µl) on pre-coated silica gel G TLC plates (0.5 mm thickness, 5×10 cm, Merk, Germany). AFB_1 (3.25 µg/ml) standard was also spotted along side the extracts and all TLC plates were developed in the solvent system $CHCl_3$: Methanol : Water (88.5:11:0.5). Subsequently the spots on TLC were viewed under UV light (365 nm). The presence of 4 fluorescing aflatoxin spots were confirmed by spraying 25% H_2SO_4 in water (Dayananda, 1990).

High performance liquid chromatography (HPLC)

Aflatoxin residues were derivatised with trifluoro acetic acid (TFA) as described by Nesheim and Brumley (1981). The derivatised aflatoxin samples and AFB1 were analyzed by a reverse phase (C-18 column) HPLC system (Agilent 1100 series, USA) 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. Three replicates were injected (10 µl) and the quantification of aflatoxin B_1 in samples was done using the peak height.

RESULTS AND DISCUSSION

A. flavus was present in all rice and paddy samples. The frequency of occurrence of *A. flavus* isolated from a sample of parboiled rice was 83.5%. The frequency of occurrence of the same fungus was 15.3% and 2.4% when isolated from two white raw rice samples collected from a farmer and a cooperative society in Kurunegala, respectively. In paddy the frequency of occurrence of *A. flavus* colonies varied from 0.2-1.3%. The identity of *A. flavus* was confirmed by IMI, UK (IMI 384871).

A significant difference could not be seen ($p>0.05$) in the mycelial dry weight in the presence of 100 ppm *C. zeylanicum* (Fig. 1) and up to 600 ppm concentration of *C. nardus* (Fig. 2). A significant difference ($P<0.05$) in the mycelial dry weight of *A. flavus* could be observed when treated with ≥1000 ppm *C. nardus* and ≥200 ppm of *C.*

zeylanicum oils. In the presence of 100 ppm to 500 ppm *C. zeylanicum* the mycelial dry weight of *A. flavus* decreased from 0.49 to 0.01 g/50 ml. The percentage inhibition of mycelial growth increased from 50 to 95 when treated with 1000 ppm to 1800 ppm of *C. nardus* oil. The MIC and MLC were 600 and 2200 ppm and 1000 and 4000 ppm for the essential oils of *C. zeylanicum* and *C. nardus*, respectively.

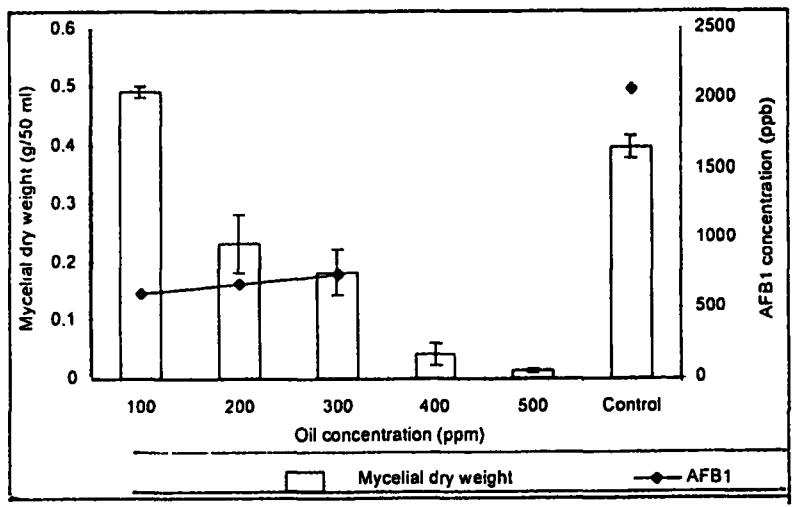

Fig. 1. The influence of the essential oil of *Cinnamomum zeylanicum* on the growth of *Aspergillus flavus* and AFB_1 production in SMKY liquid medium.
[Note: Mycelial dry weight = mean of 5 replicates and AFB_1 concentration = mean of 3 replicates].

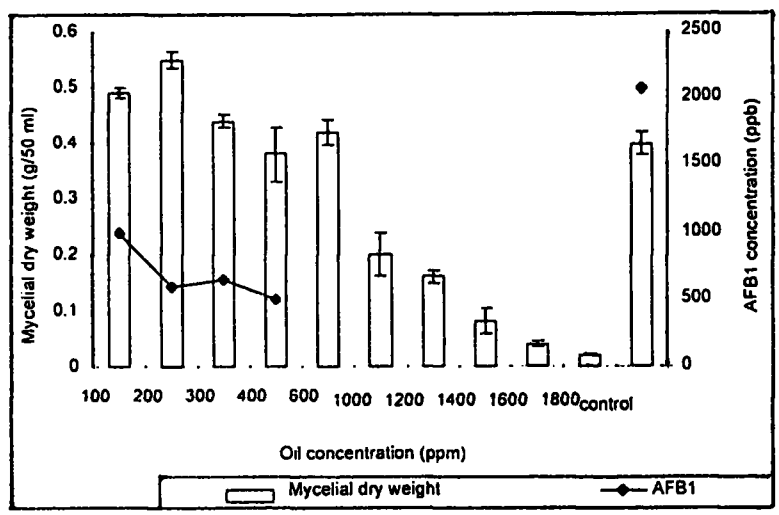

Fig. 2. The influence of the essential oils of *Cymbopogon nardus* on the growth of *Aspergillus flavus* and AFB_1 production in SMKY liquid medium.
[Mycelial dry weight = mean of 5 replicates and AFB_1 concentration = mean of 3 replicates].

The production of aflatoxin B_1 was inhibited at concentrations above 400 and 600 ppm of *C. zeylanicum* and *C. nardus* oils respectively, although the mycelial growth was not inhibited. The AFB_1 concentration of the control was significantly different when compared to the oil treatments ($P<0.05$).

The frequency of occurrence of *A. flavus* was highest on parboiled rice (83.5%). Parboiled rice is more susceptible to fungal colonization due to the high moisture level as a result of improper drying. The gelatinized endosperm also makes parboiled rice more susceptible to fungal attack (Breckenridge *et al.*, 1986).

In the present study, 1000 and 4000 ppm of *C. zeylanicum* and *C. nardus* oils were identified as MLCs against *A. flavus*, respectively. The major constituent in *C. zeylanicum* leaf oil has previously been identified as eugenol (Paranagama, 1991). Farag *et al.* (1989) have reported that the clove oil, which contains 85% eugenol, at 600 ppm could inhibit the growth of *A. parasiticus* as well as the production of aflatoxins. Eugenol is a phenolic compound and a well known antimicrobial agent, which plays an important role in plant disease resistance. Paster *et al.* (1988) have previously reported the inhibition of aflatoxin production by phenolic compounds. Therefore, the inhibitory effect of the mycelial growth and aflatoxin production could be due to the presence of eugenol in *C. zeylanicum* leaf oil.

Delespaul *et al.* (2000) have reported on the occurrence of citronellal (30%), geraniol (26%) and citronellol (15%) which are oxygenated monoterpenes as major components of the essential oil of *C. nardus*. The essential oil of *C. nardus* has been successful in suppressing the growth of *A. niger* and *A. amstelodami* at 1250 and 2500 ppm for 10 days in agar medium (Delespaul *et al.*, 2000).

TLC analysis of aflatoxin extracts showed 4 fluorescing spots under long wave UV with a blue fluorescent spot parallel to AFB_1 standard. When sprayed with H_2SO_4 all 4 spots of green and blue turned yellow under UV indicating the presence of 4 types of aflatoxins. Due to the unavailability of authentic standards, only AFB_1 in test samples were quantified. However, the total concentration of aflatoxins present in the liquid medium could be higher than the values reported here.

Aflatoxins were not present in the medium at the concentrations above 400 and 600 ppm of *C. zeylanicum* and *C. nardus*, respectively which were well below the MIC levels. This phenomenon has been reported earlier by Paster (1994) to be displayed by the extracts of pepper and cinnamon. Added alcohol extract of cinnamon at 1% level and ground cinnamon spice at 2% level completely inhibited the aflatoxin formation with a slight inhibition of the mycelial growth (Bullerman, 1974).

CONCLUSIONS

The use of spice plant material is feasible in protecting rice and other grains against fungi as they are used as flavouring agents. The fungicidal efficacy and the aflatoxin suppressing property of the essential oils of *C. zeylanicum* and *C. nardus* oils are evident from the current study. Therefore, essential oil treatments could be recommended as aflatoxin inhibitors with further investigations.

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