Studies on the Mode of Action of Neem (*Azadirachta indica*) Leaf and Seed Extracts on Morphology and Aflatoxin Production Ability of *Aspergillus parasiticus*

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Abstract

Morphological changes of a toxigenic isolate of *Aspergillus parasiticus* cultured in the presence of aqueous leaf and seed extracts obtained from neem, *Azadirachta indica* A. Juss (syn. *Melia azadirachta* L.), a potent inhibitor of aflatoxin biosynthesis, was studied. Mycelial samples obtained from *A. parasiticus* cultures exposed to an effective concentration of the leaf and seed extracts (50% v/v in culture media) produced approximately 90 and 75%, less aflatoxins respectively. Under these conditions semi-thin longitudinal and cross sections of the mycelia and vesicles showed attenuation of the cell wall at variable intervals causing deformation of the mycelium, vacuolation of the mycelial cytoplasm and vesicles. Herniation of the cytoplasmic contents which were protruding from the mycelium resulting in irregular mycelial shape. In addition, some mycelia showed a cleft between the cell wall and cytoplasm. Data obtained from microscopic observations may suggest that irreversible inhibition in aflatoxin biosynthesis by fungi due to neem extracts is partly due to mycelial cell wall damages.

INTRODUCTION

Aflatoxins are secondary metabolites produced by toxigenic strains of *Aspergillus flavus* and *A. parasiticus*. These fungi grow rapidly on a variety of natural substrates and consumption of food products contaminated with toxigenic fungi can pose serious health hazards to human and animals. Aflatoxins are well known for their hepatotoxic and hepatocarcinogenic effects (Eaton and Groopman, 1994).

Neem, *Azadirachta indica* A. Juss (syn. *Melia azadirachta* L.) is a subtropical tree native to the drier areas of Asian and African countries. Neem components have reputed value for their medicinal, spermicidal, antiviral, antibacterial, antiprotozoal, insecticidal, insect repellent, antifungal and antinematode properties (Badman et al., 1999; Khan et al., 1988; SaiRam et al., 2000). Several active substances from different parts of the tree have been identified. Extracts from different parts of the tree contain terpenoids, desactylimbin, quercetin and sitosterol (Schaff et al., 2000; Siddiqui et al., 2000).

It has been reported that addition of aqueous neem leaf extract above 10% (v/v) can effectively inhibit aflatoxin production by *Aspergillus parasiticus* and *A. flavus.* Under such conditions mycelial dry weight was unaffected. The inhibitory components in these extracts are non-volatile and influence the regulation of the synthesis of the secondary metabolic enzymes involved in aflatoxin biosynthesis (Bhatnagar and McCormic, 1988; Zeringue and Bhatnagar, 1990). Recently we have reported that inhibition of aflatoxin production in toxigenic fungi is associated with the suppression in glutathione S-transferase (GST) activity (Allameh et al., 2001). This study is focused on morphological changes of a toxigenic strain of *A. parasiticus* exposed to neem leaf and seed extracts to find cellular target(s) of neem components.

MATERIALS AND METHODS

Chemicals

Aflatoxin standards were the products of Sigma chemical Co. (St. Louis, Mo. USA). Epon-812, glutaraldehyde, formvar, lead citrate, 2,4,6-tri (dimethylamino-methyl) phenol and uranyl acetate were purchased from Taab laboratories, (UK) Osmium tetroxide was the product of Fluka (Switzerland). All other solvents and reagents were of analytical grade obtained from E. Merck (Germany).

Fungal Growth and Culture Conditions

14 isolates of *Aspergillus parasiticus* with potential ability to produce 4 major aflatoxins (AFB1, AFB2, AFG1 and AFG2) were selected among 85 isolates, which were isolated from pistachio nuts. The mycological characteristics and aflatoxin production potential of this wild-type fungal isolate was comparable to *A. parasiticus* NRRL 2999, a standard aflatoxigenic strain grown under similar conditions.

Preparation of Neem Extracts

The Forestry and Rangelands Research Institute of Iran provided neem plants, which were collected from southern parts of Iran. Fresh leaves and seeds were transferred to our laboratory and extracted as described previously (Allameh et al., 2001).

Inhibition of Aflatoxin Biosynthesis by Neem Extracts

SLS medium was inoculated with *Aspergillus parasiticus* spores (3.2×10^6 spores/ml). Then the culture medium was divided into 50-ml aliquots in 200-ml capacity flasks. Flasks were divided to different sets (3 flasks in each group). Levels of the total aflatoxins reached maximum after 96 hours at 28 ± 1°C. The ratio of SLS medium to extracts prepared in phosphate buffer was adjusted by addition of different concentrations of extract(s) to the culture media. The culture media were mixed with either leaf or seed aqueous extract at the time of spore inoculation. The final concentration of the extracts from both the sources to the culture media was calculated to be 1.56, 3.12, 6.25, 12.5, 25 and 50% (v/v). Flasks containing the same ratio of the culture media and phosphate buffer alone were used as controls. Mycelial samples obtained from cultures fed with the highest effective concentration of the extract (50% v/v) were used for morphological studies.

Measurement of Aflatoxins

One gram of fresh mycelia from each flask was taken and processed for extraction of aflatoxins using chloroform as the extraction solvent. Silica gel-GF pre-coated TLC sheets were used for analysis of aflatoxins (B1, B2, G1 and G2) produced by the fungal strain. Toxin content was measured spectrophotometrically in aflatoxin fraction eluted from silica gel according to the procedure described by Nabney and Nesbitt (1965).

Processing of Mycelia for Morphological Studies

Mycelial samples obtained from cultures grown either in 50% or no plant extract were recovered and processed for microscopic studies. At the end of the 96-h growth period when the production of aflatoxins in control flasks reached their highest levels, mycelial samples were recovered from all the flasks. The samples were processed by fixing in 3% glutaraldehyde and postfixed in 1% osmium tetroxide, dehydrating in a graded water acetone series and embedded in Epon-812. Semi-fine sections (approx. 500 nm) were cut and stained with 1% Toluidine Blue in 1% sodium borate for one minute at 80°C and used for light microscopy.

RESULTS

Effects of Neem Extracts on Aflatoxin Production

Total aflatoxins (B1+B2+G1+G2) produced by *Aspergillus parasiticus* # 14 after

96h was approximately 270 μg/flask. The production of aflatoxins after 96 hrs was inhibited by both neem leaf and seed extracts. Neem leaf extract at the lowest concentration used resulted in approximately 23% inhibition in aflatoxin production. Highest concentrations of the leaf extracts (50% v/v) resulted in >90% inhibition in aflatoxin biosynthesis. Neem leaf extract caused 10-15% more inhibition in aflatoxin production as compared to that of seed extracts.

Effects of Plant Extracts on Morphology of *A. parasiticus*

Morphological changes of the toxigenic fungus, *A. parasiticus*, grown in the presence of leaf extract are compared with control flasks with fungal culture without plant extracts is shown in Fig. 1. The longitudinal section of the fungus cultured in presence of leaf extract revealed that there are extensive vacuolation of the mycelial cytoplasm. The cell wall associated with herniation of the cytoplasmic content which protrude from the site of wall defect. There was attenuation of the cell wall at variable intervals causing deformation of the mycelium resulting in irregular mycelial shape (Fig. 1B). Some mycelia showed a cleft between the cell wall and the cytoplasm. Cross sections of the specimen showed irregular and collapsed profiles of the mycelium. The cytoplasm was dislodged and accumulation of vacuoles located underneath the cell wall caused irregularity in the cell wall (Fig. 1C). Generally, the cell was not uniform in a single profile. A small number of fungal vesicles were transversely sectioned, these vesicles were deformed and/or vacuolated (Fig. 1B).

DISCUSSION

It has been reported that decreased amount of aflatoxin synthesis by the aflatoxin producing fungi is accompanied by morphological changes (Torres et al., 1980). Non-volatile neem leaf constituents are known to potentially inhibit aflatoxin biosynthesis in *Aspergillus parasiticus* without affecting fungal growth (Bhatnagar and McCormic, 1988), whereas extracts obtained from seed inhibit both aflatoxin biosynthesis and retard fungal growth (Table 1). It has been suggested that the plant extract may influence early steps in aflatoxin biosynthesis pathways. Existence of a positive correlation between aflatoxin production and glutathione S-transferase (GST) has been established using aflatoxigenic and non-toxigenic strains (Allameh et al., 2001; Saxena et al., 1989, 1988). More recent studies showed that the mycelial cytosolic GST activity suffers profoundly when aflatoxin production is inhibited by neem leaf extract (Allameh et al., 2001).

Morphological changes in mycelia of toxigenic *A. parasiticus* treated with leaf or seed extracts indicate that effective components are present in both the preparations and that the leaf extract has relatively more inhibitory effects on aflatoxin production (Table 1). Inhibition in aflatoxin production due to neem extracts was associated with deformation of the fungal mycelia and vesicles (Fig. 1B and 1C). It seems that the mycelial cell wall is the main target of neem components. The fungal cell wall is vital to protect the cell from osmotic pressure and the disturbance in the cellular osmotic pressure can increase cell vacuolation or even death of the organism by osmotic shock (Sazykin et al., 1995). The morphological findings observed in this study are consistent with reports which documented the cell wall destruction due to other antifungal agents (Joklik and Mitchell, 1980).

In conclusion, evidences presented in this paper and our previous studies are not sufficient to establish a relationship between aflatoxin biosynthesis and cell wall integrity. Further studies needed to elucidate possible relationship between cell wall damages due to neem components and aflatoxin inhibition.

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Tables

Table 1. Effects of neem leaf and seed extracts on aflatoxin levels produced by *A. parasiticus.*

Plant extract (% v/v)	Leaf		Seed	
	Total aflatoxins (µg/50 ml)	Mycelial dry weight (g)	Total aflatoxins (µg/50 ml)	Mycelial dry weight (g)
0 (Control)	272.58±24.38	1.11±0.08	272.58±24.38	1.11±0.08
1.56	212.70±16.25	1.13±0.08	247.70±20.36	1.09±0.06
3.12	196.84±16.71*	1.11±0.09	235.91±17.83	1.05±0.08
6.25	180.68±11.22*	1.12±0.06	214.70±15.78*	0.99±0.07
12.50	117.16±8.44*	1.11±0.07	194.81±18.24*	0.98±0.07
25.00	50.05±6.46*	1.10±0.09	61.97±8.74*	0.71±0.08*
50.00	23.54±2.90*	1.10±0.07	58.66±6.22*	0.67±0.05*

Experimental details are as described in 'Materials and Methods'. Results are shown as Mean ± SEM of 3 samples carried out in duplicate. * $P < 0.05$ is considered as significantly different from the control group.

Figures

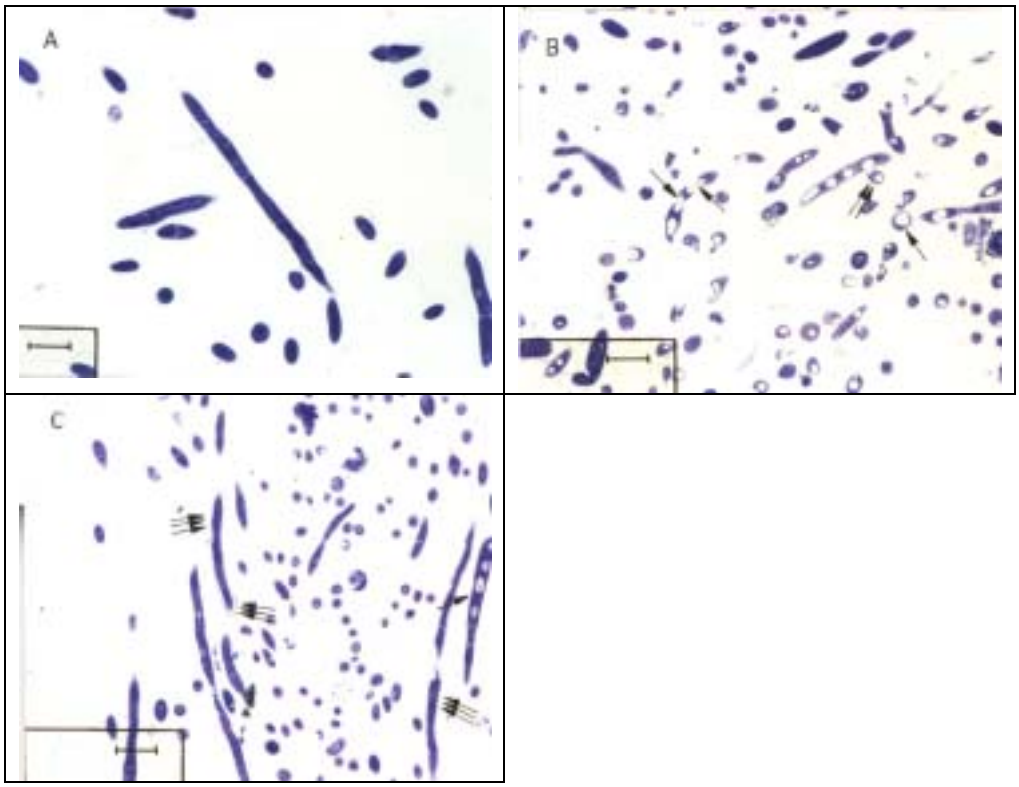

Fig. 1. Semi-thin sections of *A. parasiticus* # 14 mycelia: Effects of neem leaf and seed aqueous extracts. (Longitudinal and cross sections of mycelia stained with Toulidine blue, x1000). (A) Untreated-normal control mycelia. (B) Mycelia grown in presence of neem leaf extract (50% v/v). Vacualation of cytoplasm, attenuation of cell wall at variable intervals (single arrow), herniation and irregular thickening of cell wall (double arrow). (C) Mycelia treated with neem leaf extract. Cleft formation is indicated by triple arrows.