Ecotoxicology and Environmental Safety 72 (2009) 2115–2120

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Microscopic studies on the *Aspergillus flavus* infected kernels of commercial peanuts in Georgia

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ARTICLE INFO

Article history:
Received 12 December 2008
Received in revised form
23 March 2009
Accepted 5 April 2009
Available online 14 May 2009

Keywords:
Arachis hypogaea
Aspergillus flavus
Seed-borne nature

ABSTRACT

This article describes the use of microscopy to prove the presence of the aflatoxin producing pathogen, *Aspergillus flavus* Link ex Fries in commercially available edible peanuts in Georgia. Light microscopy in combination with electron microscopy has been used to describe the infection course established by the fungus. The alkali maceration technique used in the study was successful and sufficient to detect the kernel infection of *A. flavus* and monitor the infection percentage in edible peanuts. Percentage of infected kernel varied from one commercial outlet to another in the region. Briefly, peanut seeds from Cartersville had the highest percentage of *A. flavus* infection. Electron microscopy confirmed the seed-borne infection of this mold. Mycelium established inside the host tissues both intercellularly and intracellularly aided by active, continuous branching of young hyphae. Establishment of mycelium was also detected in the xylem vessels of roots indicative of systemic infection. Thus, edible peanuts can form an important source of inoculum and facilitate the spread of the fungus from one peanut to another in commercial outlets and elsewhere. Present study provides strong evidence that *A. flavus* can escape detection at selling points and lands in commercial outlets via edible peanuts. That these contaminated peanuts could pose public health hazards is discussed.

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1. Introduction

Aflatoxins are potent carcinogenic, mutagenic and teratogenic metabolites produced primarily by the fungal species *Aspergillus flavus* Link ex Fries and *Aspergillus parasiticus* Speare. Aflatoxin contamination of peanuts has been a serious problem since the 1960s, after it was discovered that animals had been poisoned by aflatoxin (Wilson, 1995). Peanuts and peanut products in the US are inspected using the general inspection instructions established by the Food and Drug Administration (FDA, 2006). In the United States, a 10% excess in hepatocellular cancer was observed in the Southeast, where the estimated average daily intake of aflatoxin was high compared with the North and West, areas with low aflatoxin intake (IARC, 1987) in studies that correlated geographic variation in aflatoxin content of foods with geographic variation in the incidence of liver cancer. Histological studies of host tissues invasion by *Aspergillus* sps. have centered on fungal invasion of maize (Xu et al., 2000). GUS activity was found in hyphae of *A. parasiticus* infecting the pericarp, embryo and cotyledons in peanuts indicating expression of aflatoxin biosynthetic genes in these tissues (Xu et al., 2000). Pitt et al. (1991) reported systemic infection of peanuts by *A. flavus* in soil and contaminated seeds. In an initial survey, when edible peanuts from selected commercial outlets in Kennesaw, Georgia, were incubated on media, mycelial growth and aflatoxin production by *A. flavus* were detected after incubation (Achar and Sanchez, 2006). Despite the importance of aflatoxin contamination of peanuts worldwide, the source of the fungi and their mode of entry have been less fully investigated in edible peanuts.

In this context the objective of the present investigation was to detect *A. flavus* by visual examination of edible peanuts and the percentage of viable inoculum using maceration technique in conjunction with light microscopy. Further, electron microscopy was used to confirm its mode of entry and establishment in the internal tissues of the seeds and seedlings.

2. Materials and methods

2.1. Seed-borne nature of A. flavus: light microscopy

A commercial variety of edible peanuts (Raw Jumbo Virginia) was purchased from commercial outlets in Marietta, Kennesaw and Cartersville in Georgia. Peanuts were visually examined for contamination by *A. flavus* followed by alkali maceration technique for infection of internal tissues. Maceration technique was

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0147-6513/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.ecoenv.2009.04.002

conducted according to the procedure described by Shetty et al. (1978). Ten seeds were placed in a 250 mL flask containing 50 mL of maceration solution [10% (w/v) sodium hydroxide and 0.5 g/L tryptophan blue] for 24–48 h at 27 °C. Two hundred seeds were macerated from each commercial outlet. After alkali treatment, the seeds were agitated gently in a water bath (65 °C) for 5 min. Seeds were transferred to a beaker containing 10 mL of lactophenol. Beakers were placed in a water bath (65 °C) or heated gently over a low flame until embryos were cleared. This enabled complete detachment of the embryo from the seed coat. Following maceration, presence of *A. flavus* in seed coats and embryos were detected by light microscopy. The infection rates of *A. flavus* in seed coats and in embryos were determined by light microscopy and compared to that of the visual examination.

2.2. Fixation of samples for scanning and transmission electron microscopy

Fixation of peanut samples for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) was modified following methods of Chetty (1998); Flegler et al. (1993) and Xia (2000). Contaminated peanut seeds were cut into sections, measuring not more than 1 mm × 2 mm, with a razor blade. Healthy peanut seeds were aseptically washed [rinsed once in a solution of 9 g NaCl/L DI water followed by DI water (×2)] and sectioned similarly. All sectioned samples were fixed in 3% glutaraldehyde (in 0.5 M cacodylate buffer, pH 7.2) overnight, followed by two washings (5 min each) in 0.5 M cacodylate buffer and washed twice (5 min each) in distilled water. Samples were post-fixed overnight in 1% osmium tetraoxide (aqueous) followed by two washes (5–10 min each) in distilled water. Specimens were then dehydrated through a graded ethanol series [30% for 15 min (×2), 50% for 15 min (×2), 70% for 15 min (×2), 90% for 15 min (×2) and 100% for 15 min (×3)].

2.3. Preparation of samples for transmission electron microscopy

Ethanol dehydration was followed by a final dehydration step in propylene oxide for 15 min (×3) then infiltrated with Spur's resin [2:1 propylene oxide to resin for 4 h to overnight (×2), 1:1 propylene oxide to resin (4 h to overnight), 1:2 propylene oxide to resin (4 h to overnight) and finally pure resin for 4 h to overnight (×2)]. Samples were then imbedded in fresh Spur's resin for 48 h at 60 °C (Chetty, 1998; Flegler et al., 1993; Xia, 2000). Semi-thin (0.5–1.0 μm) sections were cut on glass knives, collected onto glass slides and stained with 1% toluidine blue O. Areas of interest were then selected for ultra-thin sectioning. Ultra-thin sections (70–90 nm) were cut using a diamond knife, collected on copper grids and post-stained with 5% uranyl acetate followed by Reynolds's lead citrate stain, 10 min each.

2.4. Preparation of samples for scanning electron microscopy

Following fixation and dehydration, samples chosen for SEM were inserted, immersed in absolute ethanol, into ethanol-filled packets made from Parafilm. Both ends of the Parafilm packet were twisted shut and the entire specimen packet was plunged into liquid nitrogen (−196 °C). A pre-chilled razor blade was used to fracture the frozen specimen packets while in liquid nitrogen. The fragments were picked up with pre-chilled forceps and placed into absolute ethanol to thaw. Samples were critical point dried from CO_2 using flow monitoring and thermal regulation, mounted on aluminum stubs and sputter coated with 12 nm of gold in an Emscope SC500 coater (Humphreys et al., 1974; Hotchkiss et al., 1984) and viewed in the lower stage of a DS-130/LaB6 SEM at 10 kV.

2.5. Seedling infection

To trace the path of infection from seed to seedling, both naturally infected, artificially inoculated and control seeds were plated on Potato dextrose Agar (PDA) and incubated at 30 °C for 72 h. After incubation all seeds were sown in an environment controlled room with a temperature regime of 322 °C during the day and 232 °C during the night. Before sowing, contaminated seeds were surface sterilized using 0.1% mercuric chloride solution for 5 min followed by 5 washings in DI water. For artificial inoculation, the inoculum was obtained from contaminated peanuts. *A. flavus* spores were incubated at 30 °C for 7 days on PDA from which suspensions containing 7.5×10^5 spores/ml were made in sterile 0.05% Tween 20. Seeds were inoculated by soaking them in conidial suspensions for 1 min. Control peanuts were similarly treated except for the omission of spores. Seeds were sown in seedling trays containing steam sterilized soil (20 pound pressure for 15 min). Moisture in soil was maintained by regular watering and trays were covered with polyethylene bags to maintain 100% relative humidity. Experiments were replicated 3 times for all samples. After seedlings emergence, one and two weeks old seedlings were cut into small plant parts (roots, stems and cotyledons) and were fixed in 70% ethanol, macerated, stained with methylene blue and viewed under light microscope. Samples were also treated for SEM and TEM as described above (Sections 2.2–2.4).

3. Results

3.1. Seed-borne nature of A. flavus

Light microscopic examination revealed the presence of both mycelia and spores in the seed coat and embryos. The percentage of infection in the samples varied from one commercial outlet to another, in both these tissues. While Cartersville had the highest percentage of infection, those from Kennesaw showed the least (Table 1). However, the maceration technique in conjunction with light microscopy was found to be better in terms of detecting much higher infection rate than visual examination (Table 1) in all the samples. In addition, there was a significant correlation between infection rates of seeds observed visually with those observed under light microscopy with correlation coefficient (r) ranging from 0.80 to 0.98. The results obtained with light microscopy were also confirmed by SEM and TEM as they were able to clearly demonstrate the presence of pathogen infection structures in the tissues. Cells of the invaded host tissues were irregular and cellular architecture was completely distorted due to infection. Seed coat of healthy peanut showed undamaged seed coat, epidermis with intact cell walls and tight cell junctions between the epidermal cells (Fig. 1a). Parenchymatous cells of healthy seeds, below the epidermis, were well organized with no sign of rupture. Infected seeds showed the presence of mycelium on the damaged seed coat and a fractured discontinuous epidermis (Fig. 1b) with loose and broken cell junctions between the epidermal cells. Parenchymatous cells were also

Table 1
Percentage of infection in edible peanuts infected by *Aspergillus flavus*.

Peanut samples[a]	No. of seeds examined/ retail outlets	Infection percentage (%)[b]		
		Visual examination	Light microscopy	
		Seeds coats	Seed coats	Embryos
Kennesaw	200	15.00^{A}	20.95^{A}	35.02^{A}
Marietta	210	17.28^{A}	22.18^{A}	43.90^{AB}
Cartersville	205	30.11^{B}	32.80^{B}	50.60^{BC}
Matrix of linear correlation coefficient (r)[c]				
Visual examination: infection % of seed coat		1.0000	0.7990*	0.9889**
Light microscopy: infection % of seed coat		0.8080*	0.9010	0.8979**
Light microscopy infection % of embryos		0.9999**	0.9889**	1.0000

[a] Ten samples from 3 different commercial outlets from each location in Georgia.
[b] Percentages within column followed by the same letter (upper case) do not differ at $P = 0.01$ according to Turkey–Kramer's multiple comparison test.
[c] Correlation coefficients followed by * or ** indicate statistical significance at $P = 0.05$ or $P = 0.01$, respectively.