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Aspergillus parasiticus Growth and Aflatoxin Production on Black and White Pepper and the Inhibitory Action of Their Chemical Constituents

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Aspergillus parasiticus **Speare NRRL 2999 growth and aflatoxin production in black and white pepper and the penetration of the fungus in black pepper corn over various incubation periods were studied. Also, the effects of piperine and pepper oil on growth and aflatoxin production were studied. Under laboratory conditions, black and white pepper supported aflatoxin production (62.5 and 44 ppb (ng/g), respectively) over 30 days of incubation. Fungal growth measured in terms of chitin was considerably less in white pepper than in black pepper. A histological study of black pepper corn showed the fungus penetrating up to the inner mesocarp and establishing itself in the middle mesocarp. Piperine and pepper oil were found to inhibit fungal growth and toxin production in a dose-dependent manner. Thus, both black and white pepper could be considered as poor substrates for fungal growth and aflatoxin production.**

Black pepper (*Piper nigrum* L.), an important spice, accounts for about 35% of the world trade in spices. The white pepper obtained from black pepper by removing the outer pericarp of the berries accounts for about 25% of the world production of pepper (16). The contamination of pepper by aflatoxigenic fungi (4, 7, 14, 22, 23) has been of increasing concern in international trade circles. The incidence of *Aspergillus flavus* and the occurrence of aflatoxin in black and white peppers have been documented (2, 19, 21). Seenappa and Kempton (20) demonstrated the substrate suitability of black pepper for aflatoxin production by *A. flavus* under artificial conditions. On the contrary, the failure to detect aflatoxin in a number of black and white pepper samples (18) had led to the assumption that pepper may not be a suitable substrate for aflatoxin production. Furthermore, the inhibitory effects of chloroform extract and pepper powder on the growth and aflatoxin production of *A. flavus* and *Aspergillus parasiticus* have been demonstrated (10, 12). Bullerman et al. (3) demonstrated the inhibitory effects of spices, such as cinnamon and clove, on fungal growth and aflatoxin production. The inhibitory action was found to be due to their chemical components, such as cinnamic aldehyde and eugenol, respectively. The present study was planned with the following objectives: (i) to reevaluate and compare the substrate suitability of black and white pepper for *A. parasiticus* Speare growth and aflatoxin production; (ii) to observe fungal penetration and growth in pepper corn through a histological study; and (iii) to determine the inhibitory effects of piperine and essential oil of pepper on *A. parasiticus* growth and aflatoxin production.

MATERIALS AND METHODS

Black and white pepper samples (2 kg each) were obtained from an Indian exporter. Samples (50 g) of each were placed in 500-ml Erlenmeyer flasks in replicates and rehydrated by the addition of 20 ml of distilled water to each flask. After being autoclaved at 121°C under a pressure of 15 lb/in^2 for 15 min, samples were each inoculated with 3 ml of spore suspension (ca. 4.5×10^6 spores per ml of 4-day-old *A. parasiticus* Speare NRRL 2999 cultured on PDA slants). Uninoculated samples served as the controls. Three sets were incubated at 28 ± 1°C for 7, 15, and 30 days, respectively, and were sprayed with 95% ethanol and powdered after being dried overnight at 80°C.

To study the inhibitory action of piperine and pepper oil, 50 ml of yeast extract sucrose broth (6) was dispensed into 250-ml Erlenmeyer flasks in duplicates and autoclaved at 121°C for 15 min. Before autoclaving, piperine dissolved in dimethyl sulfoxide was added to give the following concentrations: 0, 10, 100, 1,000, and 10,000 ppm (μg/ml). Pepper oil emulsified with a few drops of Tween 80 was added to yeast extract sucrose broth separately, after autoclaving, to give concentrations of 0, 100, 250, and 500 ppm. The inoculum was prepared as described above and used at the rate of 1 ml per flask. The flasks were incubated at 28 ± 1°C for 7 days. Pure crystalline piperine was secured from the Central Food Technological Research Institute, Mysore, India, and pepper oil was obtained by steam distillation of black pepper powder.

Estimation of fungal growth. Fungal growth was measured by the method of Ride and Drysdale (17) for estimating filamentous fungal biomass in infected plant tissue by chitin concentration. Samples of 25 mg of ground black pepper and 100 mg of white pepper were homogenized in acetone to remove fat. The homogenate was centrifuged, and the supernatant was removed. The residue was washed with water and recentrifuged. The washings were discarded, and the residue was treated with concentrated KOH for 1 h at 130°C. The hydrolyzed material was washed progressively with ethanol and water. Washing with water had to be repeated 4 times to eliminate pigments and oleoresin, which posed the problem of precipitation when treated with $FeCl_3$ solution in the process. The conversion factor for glucosamine into dry mycelia was determined by growing *A. parasiticus* in liquid medium (1) for 7 days and estimating the glucosamine colorimetrically (17).

To measure the fungal growth in liquid medium after incubation, the cultures were given a brief heat treatment by being autoclaved at 121°C for 30 s to kill the spores and

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TABLE 1. *A. parasiticus* Speare NRRL 2999 growth and aflatoxin production on black and white pepper[a]

Pepper at day of incubation	Mycelial dry wt (mg/g)	Aflatoxin B_1 (μg/kg)	Aflatoxin B_1 in mycelia (dry wt) (μg/mg)
Black			
7	10.40 ± 0.64	52.50 ± 3.50	5.05 ± 1.17
15	19.50 ± 1.06	55.50 ± 0.85	2.85 ± 0.17
30	21.90 ± 1.18	62.50 ± 7.50	2.85 ± 0.20
White			
7	3.50 ± 1.00	21.90 ± 5.43	6.18 ± 0.85
15	4.70 ± 0.26	21.30 ± 3.46	4.49 ± 0.45
30	5.00 ± 0.35	44.30 ± 7.06	8.86 ± 1.47

[a] Values given are mean ± standard deviation.

vegetative mycelium. The mycelial mats were separated from the residual culture broth, washed with distilled water, and dried at 80°C for 16 h. The weight of the dried mycelial mat was then determined.

Histological study. Infected pepper corns from samples incubated for different periods as described above were fixed and sectioned as per the standard procedure (11). Series of sections were taken with a rotary microtome and stained with Pianese III B as described by Conn et al. (5). Permanent mounts were prepared with Canada balsam for microscope observations.

Estimation of aflatoxin. The powdered samples were analyzed for aflatoxin by the modified method of Suzuki et al. (21). In the modified method, a layer of neutral alumina (5 g activated at 110°C for 2 h) was included between the upper anhydrous Na_2SO_4 layer and the silica gel layer in column chromatography. Aflatoxin B_1 was quantitated densitometrically (15).

The aflatoxin from the culture filtrate was extracted with chloroform, evaporated to dryness, dissolved in a known volume of benzene-acetonitrile (98:2 [vol/vol]), and chromatographed by thin-layer chromatography. Quantitation was done densitometrically (15).

RESULTS AND DISCUSSION

In black pepper, the fungal growth was almost doubled after 15 days of incubation when compared with that after 7 days, but further growth of the fungus between 15 and 30 days was minimal (Table 1). However, there was a gradual increase in toxin production from 7 to 30 days of incubation, indicating that fungal growth is not correlated to toxin production. It is evident from the histological study that the fungus penetrates the epicarp during the first 7 days and reaches the middle mesocarp within 15 days. It gets estab-

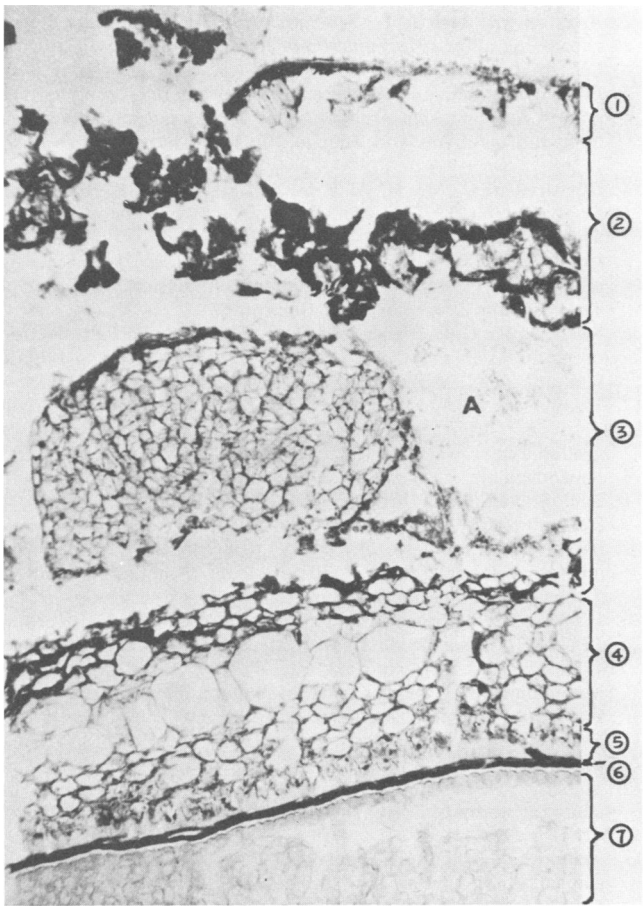

FIG. 1. Cross-section of a black pepper corn artificially infected with *A. parasiticus*, showing the extent of fungal invasion after 15 days of incubation (×100). 1, Epicarp plus hypoderm; 2, outer mesocarp; 3, middle mesocarp; 4, inner mesocarp; 5, endocarp; 6, spermoderm; 7, perisperm; A, cavity containing conidia.

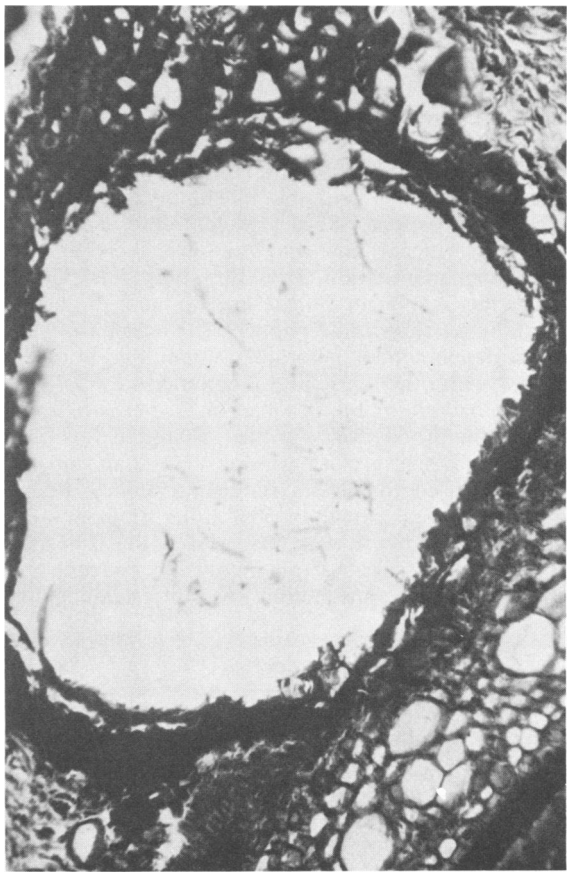

FIG. 2. Cross-section of a black pepper corn artificially infected with *A. parasiticus* after 30 days of incubation, showing a cavity in the middle mesocarp containing conidia (×100).

TABLE 2. Inhibitory action of piperine on *A. parasiticus* Speare NRRL 2999 growth and aflatoxin production[a]

Piperine added (ppm)	Mycelial dry wt (mg/ml)	Aflatoxin B_1 (μg/ml)	Aflatoxin G_1 (μg/ml)
0	47.84 ± 1.73	39.00 ± 4.24	14.50 ± 0.71
10	28.41 ± 0.35	13.80 ± 0.28	3.80 ± 0.28
100	28.09 ± 0.83	7.60 ± 1.70	3.20 ± 0.28
1,000	24.08 ± 0.00	4.40 ± 0.57	2.35 ± 0.07
10,000	21.29 ± 1.51	1.45 ± 0.21	0.4 ± 0.00

[a] Values given are mean ± standard deviation.

lished in the cavities that appear to be resulting from the degeneration of vascular tissues with 30 days of incubation (Fig. 1 and 2). With scanning electron microscopic studies, Seenappa and Kempton (20) observed that *A. flavus* colonized more on ridges of the pepper corn, whereas Neumayer and Forstmeier (13) found the spores distributed in the outer half of the pericarp.

In white pepper, the fungal growth was low over 7, 15, or 30 days of incubation (Table 1). The endocarp, formed of thick-walled, beaker-shaped cells, could offer a physical barrier to the fungal penetration, thereby confining growth to the mesocarp.

Toxin production at 7 and 15 days of incubation was almost equal. However, it doubled during 30 days of incubation, and there was no correlation between fungal growth and toxin production, as in the case of black pepper.

The amount of aflatoxin found in black pepper under laboratory conditions during the present study was similar to that observed by Seenappa and Kempton (20). However, the amount of aflatoxin found in white pepper under laboratory conditions in the present study was considerably less than that reported by Flanningan and Hui (7). It may be pertinent to mention here that aflatoxin-like fluorescent substances are present in pepper, and if sufficient care is not taken to remove these during analysis, they are likely to interfere with aflatoxin quantitation. From this study, it is evident that black pepper supports fungal growth and production of aflatoxin better than white pepper. The amount of aflatoxin found in pepper under laboratory conditions is much less compared with other agricultural commodities (9).

Piperine had an inhibitory effect on *A. parasiticus* growth and aflatoxin production. At the 1,000-ppm level, a 50% inhibition of fungal growth was observed, and above that level it appeared to have a fungistatic effect. Pepper oil inhibited the fungal growth by 50% at the 100-ppm level, and scanty mycelial growth was observed at the 250- and 500-ppm levels. The effect of an increasing concentration of piperine on aflatoxin production was quite significant (Table 2). At the 10,000-ppm level, about a 98% reduction in aflatoxin production was observed. Pepper oil stimulated the aflatoxin production at the 100-ppm level. However, at higher concentrations, due to the inhibition of fungal growth, aflatoxin production was reduced to a negligible level (Table 3). Black pepper contains 2 to 8% (20,000 to 80,000 ppm) piperine and 2 to 4% (20,000 to 40,000 ppm) essential oil (8). However, their distribution is restricted to cells such as oleoresin cells and oil cells, respectively, in pepper corn. As such, they may not be easily accessible to the growing fungus in vivo; hence, the effect is minimal when compared with that in vitro. Thus, our data indicate that the inhibitory action of black pepper on fungal growth and aflatoxin production as reported by Hitokoto et al. (10) and Mabrouk and El-Shayeb (12) could be due to its main pungent principle, piperine, and the volatile essential oil which is responsible for its aroma.

TABLE 3. Inhibitory action of pepper oil on *A. parasiticus* Speare NRRL 2999 growth and aflatoxin production[a]

Pepper oil added (ppm)	Mycelial dry wt (mg/ml)	Aflatoxin B_1 (μg/ml)	Afatoxin G_1 (μg/ml)
0	49.15 ± 0.92	41.00 ± 4.24	15.80 ± 0.28
100	31.95 ± 1.63	44.50 ± 0.71	27.50 ± 3.54
250	11.25 ± 0.35	Tr	Tr
500	9.00 ± 0.00	Nil	Nil

[a] Values given are mean ± standard deviation.

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