Production of Aflatoxins by *Aspergillus flavus* Cultured on Flue-Cured Tobacco

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Under favorable growth conditions, *Aspergillus flavus* can produce aflatoxins on flue-cured tobacco leaves.

During the preparation of flue-cured tobacco for market, *Aspergillus flavus* Link occasionally invades the tissue and can be isolated from cured leaves offered for sale at tobacco warehouses (9) and from damaged stored tobacco (7). However, this fungus has not been isolated from tobacco leaves just before and immediately after flue-curing on the farm (8).

The purpose of this study was to determine whether aflatoxins could be produced on flue-cured tobacco inoculated with an *A. flavus* isolated from damaged tobacco and to develop a method by means of which aflatoxin would be extracted and separated from scopoletin (1, 4, 6). The presence of aflatoxin constitutes a health hazard (5) and should be of particular importance when tobacco is consumed by methods other than smoking.

Flue-cured tobacco leaves from all positions on the stalk, and practically free of the brown-spot disease (caused by *Alternaria tenuis*), were cured as previously described (8), cut into ribbons 1 mm wide and of various lengths, and stored at 15.8 to 17.1% moisture content (wet weight) until used. This shredded tobacco was inoculated and moistened to approximately 60% moisture content, to insure *A. flavus* growth, by adding 50 ml of an aqueous spore suspension (1,000,000 spores/ml) of *A. flavus* to a flask containing 150 g of tobacco. The flasks were stoppered with cotton plugs and incubated at room temperature (23 to 28 C) for 2 months; they were shaken occasionally to break up mycelial mats. A second experiment was undertaken to confirm the observations of the first one and to indicate the influence of aeration on the production of aflatoxin. The following changes were made in the sample treatment. The flasks were inoculated and incubated for 4 days to allow establishment of the fungus before application of the treatments. The treatments were:

- continuous aeration, standing without air circulation, and replacement of air by flushing thoroughly with an N2 stream and then sealing the system to prevent gas leakage. The air and N2 gases were moistened by passing them through distilled sterile water before they entered the flasks.

![Schematic reproduction of thin-layer chromatographic plate showing separation of aflatoxin B1 and G1 and fluorescent compounds isolated from tobacco. Symbols: 1, standard aflatoxin B1 and G1; 2, extract purified by the Tso and Sorokin modification of the Pons method; 3, extract purified by modification of the Pons method; S, scopoletin. Coating: silica gel G-HR. Solvent: chloroform-methanol (97:3).](Fig. 1)

Aflatoxin was determined by the following modification of the method of Pons et al. (4). Samples were extracted with 300 ml of 70% acetone and filtered; approximately 220 ml was collected. To this filtrate was added 5 g of insolu-
polyvinylpyrrolidone (Polyclar At; General Aniline and Film Corp., New York, N.Y.). The mixture was shaken for 5 min and filtered; 180 ml was collected. This volume was used for extract purification. Activated silica gel Mallinkrodt CC-7 (100 to 200 mesh) was used for column chromatography. After addition of the extract, the column was washed, in sequence, with 100 ml each of ethyl ether and hexane and eluted with the methanol–chloroform (3:97) mixture.

Thin-layer chromatographic analysis (silica gel G-HR and chloroform-methanol, 97:3 as solvent) of inoculated, shredded, flue-cured tobacco leaf samples, using the above extract, resulted in fluorescent spots corresponding to aflatoxins B₁, B₂, G₁, and G₂ (Fig. 1). The identity of aflatoxin B₁ was confirmed by the derivative methods of Andrellos and Reid (2), and in all cases the tobacco aflatoxin B₁ derivatives matched the derivatives made from standard aflatoxin B₁ (Fig. 2). The growth of *A. flavus* was continuous during the 2 months of incubation, and final yields of aflatoxin B₁ were greater than 100 μg/kg from each of the four samples. Uninoculated tobacco examined in the same way contained no aflatoxin or scopolin.

Thin-layer chromatographic analysis of the samples from the second experiment was performed using four solvent systems: benzene–methyl–acetic acid, 80:7:10; toluene–ethyl acetate–formic acid, 50:40:10; chloroform–methanol, 97:3; and chloroform–acetone, 90:10. All samples shown to contain spots corresponding to aflatoxin were confirmed by the additional solvent systems. Aeration plays an important role in the production of aflatoxin on tobacco (Table 1). When adequate aeration was present, a predominance of the samples contained significant quantities of aflatoxin. However, under inadequate aeration a majority of the samples produced no aflatoxin.

I appreciate the generous assistance of Ronald E. Welty in supplying the inoculated tobacco samples.

### Table 1. Influence of aeration on the production of aflatoxin in flue-cured tobacco

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of samples</th>
<th>Percent positive</th>
<th>Mean aflatoxin content (μg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeration</td>
<td>4</td>
<td>75</td>
<td>203</td>
</tr>
<tr>
<td>No aeration</td>
<td>18</td>
<td>33</td>
<td>57</td>
</tr>
<tr>
<td>N₂</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

![Fig. 2. Schematic reproduction of thin-layer chromatographic plate showing separation of derivatives of standard aflatoxin B₁ and tobacco aflatoxin B₁. Symbols: S, standard aflatoxin B₁; T, tobacco aflatoxin B₁; A, acetic acid derivatives; F, formic acid derivatives; TF, trifluoroacetic acid derivatives; B₁, unreacted standard aflatoxin B₁. Coating: silica gel G-HR. Solvent: chloroform:acetone (9:1).](image-url)