Tremorgenic indole metabolites and aflatoxins in sclerotia of *Aspergillus flavus*: an evolutionary perspective

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Received June 5, 1981


Isolates of *Aspergillus flavus* Link from both cool and warm latitudes were cultured on potato dextrose agar containing yeast extract to identify sclerotia-producing strains. Chloroform-MeOH extracts of sclerotia were analyzed for the presence of aflatoxins and major indole metabolites (e.g., cyclopiazonic acid, aflatrem, and dihydroxyaflavinine). Aflatoxin is reported from sclerotia of *A. flavus* for the first time. Cyclopiazonic acid was detected primarily in sclerotia of isolates from warmer latitudes. Aflatrem and dihydroxyaflavinine were detected in sclerotia from 85% of the strains examined. These metabolites are associated with the sclerotial stage of the life cycle, because neither were detected in extracts of the culture medium and mycelium of Petri dish cultures from which all the sclerotia were removed. Geographic variation and intrafungal allocation of these toxic compounds in *A. flavus* are examined from the evolutionary ecologist’s perspective of selective forces shaping the chemical defense systems of fungi.

Materials and methods

Fungal strains and culture collections

A general survey was conducted of approximately 100 *A. flavus* strains for their ability to produce sclerotia when inoculated (three points) onto potato dextrose agar (PDA).
containing 0.5% yeast extract (YE) in Petri dishes. Dishes were incubated in the dark at 25°C for 21 days, an interval that allowed for maturation of all sclerotia produced. From these, we selected sclerotial-producing isolates from warmer (29 strains) versus cooler (19 strains) latitudes (Table 1). All sclerotia produced in one Petri dish (0.3–0.7 g) were harvested using a spatula and transferred to a Braun cell homogenizer bottle containing 20 mL distilled water. To facilitate the separation of sclerotia from contaminating pieces of agar, conidia, and mycelium, the bottle and its contents were cooled in a stream of liquid CO₂ and shaken for 20 s using a Braun cell homogenizer (Braun Scientific, Inc.). In preliminary trials, we found that a 20-s interval was sufficient to accomplish our objective without substantial damage to the sclerotium rind. Because the sclerotia settled rapidly in water, contaminating fragments could be removed easily through successive decanting. Sclerotia then were transferred to screw-capped vials, freeze-dried, and stored at 5°C until chemical analyses could be performed (<3 months).

**Extraction, purification, and chemical analyses of major secondary metabolites**

Sclerotia or the mycelium and culture medium were ground with a porcelain mortar and pestle and extracted with chloroform–MeOH (3:1, v/v). The extracted material then was filtered and evaporated to dryness. The extracts then were redissolved in 500 μL chloroform for thin-layer chromatography (TLC) analyses. Twenty microfilters were applied to precoated silica gel 60 F-254 TLC plates (5 × 10 cm) and developed in chloroform–acetone (93:7, v/v) and toluene–ethyl acetate–formic acid (5:4:1, v/v/v). Indole metabolites under study were detected on TLC plates by first spraying with 1% ethanolic dimethylaminobenzaldehyde followed by spraying with 50% ethanolic H₂SO₄ and gentle heating to bring out only the indole-containing metabolites (limits for detection of indoles ca. 1 μg). Color responses intensified, with time, at room temperature. Aflatoxins were detected under long-wave ultraviolet (UV) light prior to spraying and heating for indole metabolites. Identifications were made by comparing R_f values on both TLC systems and color response relative to standards. Because it was not practical to isolate pure metabolites for identification in all cases, we selected a known toxigenic strain of *A. flavus* (NRRL 3251) and isolated the metabolites under study in pure form for conclusive identification by comparisons of infrared (IR), UV, mass spectra, ¹³C NMR, and ¹H NMR of standards (Cole *et al.* 1981). In this way we were able to identify the metabolites in the survey by TLC, and then verify that compounds of similar R_f in TLC from one isolate were actually aflatoxin, cyclopiazonic acid, aflatrem, and dihydroxyaflavin.

### Results and discussion

The distribution of sclerotial metabolites from among those kinds under study are presented in Table 1. Aflatrem (Gallagher, Clardy *et al.* 1980) and dihydroxyaflavinine (Gallagher, McCabe *et al.* 1980; Cole *et al.* 1981) were detected in sclerotia of the great majority (>85%) of isolates examined. Aflatrem has been linked with observations of trembling, convulsions, and even death in mice to which it was administered orally with crude extracts of moistened cracked corn or 14-day-old agar plate cultures on which a heavily sclerotial-producing strain of *A. flavus* (NRRL 500 = QM 6738) was grown (Wilson and Wilson 1964; Wilson 1971; Gallagher, Clardy *et al.* 1980). Detectable quantities of the tremorgenic toxin could be extracted from the sclerotia but not from the conidia of this strain (Wilson and Wilson 1964).

Our study was, by design, limited to the major indole metabolites extracted from relatively small samples of *A. flavus* sclerotia. Because aflatrem was detected in nearly all of the sclerotia examined and because this compound represents the most logical distal product in the biosynthesis of this group of fungal metabolites (Cole 1981), we believe that a more thorough examina-

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**Table 1. Number of isolates producing aflatoxins, cyclopiazonic acid, aflatrem, and dihydroxyaflavinine in sclerotia of *Aspergillus flavus***

<table>
<thead>
<tr>
<th>Detection of metabolite*</th>
<th>Aflatoxins</th>
<th>Cyclopiazonic acid</th>
<th>Aflatrem</th>
<th>Dihydroxyaflavinine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B₁ B₂ G₁ G₂ P†</td>
<td>Warmer latitudes‡</td>
<td></td>
<td>Cooler latitudes§</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>12 3 1 — —</td>
<td>4</td>
<td>15 18 17 19 17</td>
</tr>
<tr>
<td>ND</td>
<td></td>
<td>17 26 28 29</td>
<td>25 27</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>4 1 2 — —</td>
<td>2 5</td>
<td>14 16</td>
</tr>
<tr>
<td>ND</td>
<td></td>
<td>15 18 17 19</td>
<td>14 17</td>
<td>5 3</td>
</tr>
</tbody>
</table>

*Present (+); not detected (ND).
†Polar compound (P).
‡Warmer latitudes (29 isolates): Brazil (4); Ecuador (1); Puerto Rico (2); Florida (1); Pacific Island (1); Hawaii (6); Thailand (1); Malaysia (1); New Guinea (1); Liberia (1); Gold Coast, West Africa (1); South Africa (5); India (1); Pakistan (3).
§Cooler latitudes (19 isolates): Ontario (1); New York (1); Wisconsin (1); Iowa (6); Illinois (4); Utah (1); Montana (1); Michigan (1); Connecticut (1); Turkey (2).

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*CAN. J. BOT. VOL. 60, 1982*
compounds were also detected in the sclerotia. Bennett origin very similar to that of ergot alkaloids. Culture medium of those isolates tested where the same contrasted with isolates from cooler latitudes (5/19 interesting to note that numerous tropical plant species lates from warmer latitudes (25/29 isolates = 82%) as Westling, was commonly detected in sclerotia of iso­
tion of a larger quantity of type tremorgenic compounds. It is interesting to note that numerous tropical plant species are recognized for their ability to produce kinds and quantities of alkaloids in what is generally regarded to be an evolutionary response to the larger number of potential predators (i.e., insects) in tropical ecosystems (Levin 1976).

Cyclopiazonic acid, originally isolated by Holzapfel (1968) as a metabolite of Penicillium cyclopium Westling, was commonly detected in sclerotia of isolates from warmer latitudes (25/29 isolates = 82%) as contrasted with isolates from cooler latitudes (5/19 isolates = 26%). According to Floss (1976), the structure of cyclopiazonic acid suggests a biosynthetic origin very similar to that of ergot alkaloids. It is interesting to note that numerous tropical plant species are recognized for their ability to produce kinds and quantities of alkaloids in what is generally regarded to be an evolutionary response to the larger number of potential predators (i.e., insects) in tropical ecosystems (Levin 1976).

Aflatoxins, represented primarily by aflatoxin B1, were also detected more frequently in sclerotia from warmer latitudes (12/29 = 41%) as compared with those cooler latitude isolates (4/19 = 21%). However, the data are only suggestive because chi-square tests of frequencies in Table 1 did not yield a significant result at the 5% level. This represents the first report of aflatoxin being detected in A. flavus sclerotia. Earlier studies demonstrated the presence of aflatoxins in conidia of two strains of Aspergillus parasiticus (NRRL 2999; NRRL 3000) (Hesseltine et al. 1966).

We next wanted to determine whether the synthesis of these metabolites occurs only within the sclerotium. In a separate growth experiment, the same chemical analyses were performed on purified extracts of mycelium and agar medium of selected Petri dish cultures from which sclerotia had been harvested and analyzed separately (Table 2). Strains were chosen because their sclerotia contained different metabolite combinations. Aflatoxins and cyclopiazonic acid were detected in the mycelium—culture medium of those isolates tested where the same compounds were also detected in the sclerotia. Bennett et al. (1979) found no relationship between aflatoxin-

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Aflatoxins</th>
<th>Cyclopiazonic acid</th>
<th>Aflatrem</th>
<th>Dihydroxyaflavinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRRL 6551</td>
<td>+(+)</td>
<td>+(+)</td>
<td>-(+)</td>
<td>-(+)</td>
</tr>
<tr>
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<td>-(-)</td>
<td>-(+)</td>
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</tr>
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<td>-(+)</td>
</tr>
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<tr>
<td>NRRL 6556</td>
<td>-(-)</td>
<td>-(-)</td>
<td>-(+)</td>
<td>-(+)</td>
</tr>
</tbody>
</table>

*Isolates examined (6): NRRL 6551 soil, India; NRRL 6552 pine sawfly, Wisconsin; NRRL 6553 soil, Pakistan; NRRL 6554 soil, Utah; NRRL 6555 corn, South Africa; NRRL 6556 soil, Illinois.

†Detection of metabolite in sclerotia in parentheses.

and sclerotium-producing ability among a total of 14 strains of A. flavus and A. parasiticus. In their study, aflatoxin B1 was measured by harvesting the mycelium of shake cultures consisting of a liquid medium described by Adye and Mateles (1964), and sclerotia were quantified from the agar surface (PDA + YE) of Petri dish cultures. Our results, based on separate analyses of sclerotia and solid substrate culture medium containing mycelium, provide additional evidence that sclerotium-producing strains may or may not produce aflatoxins. Neither aflatrem, dihydroxyaflavinine, nor any other related neutral indole metabolite was detected in the mycelium—culture medium from which sclerotia containing these metabolites were harvested.

Because sclerotia are commonly recognized for their nutrient-storage, dormancy, and survival properties, efforts to assess their adaptive significance have usually centered on their resistance to stressors in the physical environment, such as desiccation (Willets 1971; Coley-Smith and Cooke 1971). We would argue that the survival of sclerotia should also be examined in connection with the means by which these structures reduce the negative effects of predation. Predation of sclerotia by other fungi (mycoparasites) is now receiving the increased attention of mycologists charged with developing strategies to control sclerotium-forming plant pathogenic fungi (Lumsden 1981). Janzen (1977) has developed a logical argument that toxigenic properties of fungal secondary metabolites should be assigned the same ecological function as that of higher plant chemical defenses. Sclerotia are critical to the survival of plant pathogens such as Claviceps spp. (Willets 1972). One might expect that seed-eating rodents and birds regularly encounter the mature sclerotia of Claviceps spp., as would an array of soil- and litter-inhabiting invertebrates. The ergot alkaloids and related indole metabolites (Floss 1976) found concentrated in the sclerotia of Claviceps spp. probably function in protecting the sclerotium from predation, an ecological role attributed to alkaloids in vascular plant tissues (Robinson 1979).
Smith and Galbraith (1971) recognized a connection between the development of certain specialized structures that serve a survival function and the accumulation of secondary metabolites. Sclerotia of different fungal taxa are likely to be confronted with many of the same potential predators. Such fungi could benefit from the same classes of secondary defensive compounds, and one might anticipate finding evolutionary convergence in pathways leading to their biosynthesis. This could explain why similar paspalinine-type metabolites can be isolated from sclerotia of unrelated fungal taxa.

An individual’s fitness is essentially determined by the number of surviving offspring produced during its lifetime. Rhoades (1979) observed that chemical defenses are costly in terms of fitness to organisms because secondary substances are the end product of energy-demanding synthesis (Solomon and Crane 1970), and it is reasonable to assume that there has been positive selection for their production. Common sense predicts that intraorganism defenses should be allocated in direct proportion to the risk of the particular tissue and the value of that tissue in terms of fitness loss to the organism resulting from attack on that tissue. Ecologists have theorized that different types of secondary compounds are differentially suited to defense of different plant parts, even though attempts to predict the selective factors that drive the evolution of intraplant distribution of chemical defenses are not always successful (Janzen 1973; McKey 1979). Our results show that unique secondary metabolites are associated with sclerotia but not vegetative mycelia or conidia of A. flavus. We believe that sclerotium-producing fungi offer chemical ecologists an excellent opportunity to conveniently study the distribution and diversity of secondary metabolites in different parts of the same organism.

Acknowledgement

We thank Amy Axt for technical assistance.