Molecular Mechanisms of Insecticide Resistance
Diversity Among Insects

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Resistance to aflatoxin B₁ in *Drosophila melanogaster* is conferred by at least two chromosomes and cytoplasmic factors, and it is thought that unspecific monooxygenases may be involved. Resistance to α-amanitin in *D. melanogaster* is due to an altered target site, RNA polymerase II. Relative resistance to aflatoxin B₁ and griseofulvin in *Spodoptera frugiperda* is due to lower rates of activation and higher rates of detoxification compared to *Helicoverpa zea*. Fungus-feeding larvae of *Carpophilus hemipterus* are able to hydrolyze a model trichothecene substrate at about 10-fold the rate of *H. zea* and *S. frugiperda*.

Mycotoxins are secondary metabolites produced by fungi that are toxic to animals, including humans. They represent just a small portion of the many secondary metabolites produced by fungi (1,2). Most mycotoxins are produced by molds in the genera *Aspergillus*, *Penicillium*, and *Fusarium*. Some representative and their effects are listed in Table I, and corresponding structures are shown in Figure 1.

Unlike plant secondary metabolites, the recognition that fungal secondary metabolites such as mycotoxins can act as defensive substances has occurred only recently (3). Nevertheless, the effects of several mycotoxins on insects have been studied to some degree (see reviews 4,5). Studies on insects have been prompted by desires to examine the defensive capabilities of mycotoxins, to test insects as alternative bioassay indicators, and to search for new insecticides or novel bioactive metabolites that provide leads for new insecticides.
Figure 1. Representative mycotoxins: a. aflatoxin B$_1$, b. ochratoxin A, c. griseofulvin, d. penitrem A, e. deoxynivalenol, f. diacetoxy-scirpenol, g. T-2 toxin, h. α-amanitin.
### Table I. Representative Mycotoxins and Their Effects

<table>
<thead>
<tr>
<th>Mycotoxin</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus</td>
<td></td>
</tr>
<tr>
<td>aflatoxins</td>
<td>hepatotoxic, carcinogenic</td>
</tr>
<tr>
<td>sterigmatocystin</td>
<td>carcinogenic</td>
</tr>
<tr>
<td>ochratoxin A</td>
<td>nephrotoxic, carcinogenic</td>
</tr>
<tr>
<td>Penicillium</td>
<td></td>
</tr>
<tr>
<td>citrinin</td>
<td>hepatotoxic</td>
</tr>
<tr>
<td>griseofulvin</td>
<td>carcinogenic, teratogenic</td>
</tr>
<tr>
<td>patulin</td>
<td>toxic, carcinogenic</td>
</tr>
<tr>
<td>rubratoxins</td>
<td>hepatotoxic</td>
</tr>
<tr>
<td>penitrems</td>
<td>tremorgenic</td>
</tr>
<tr>
<td>penicillic acid</td>
<td>carcinogenic</td>
</tr>
<tr>
<td>Fusarium</td>
<td></td>
</tr>
<tr>
<td>T-2 toxin</td>
<td>dermal necrosis, hemorrhagic</td>
</tr>
<tr>
<td>deoxynivalenol</td>
<td>emetic, nephrotoxic</td>
</tr>
<tr>
<td>diacetoxydialenol</td>
<td>dermal necrosis, hemorrhagic</td>
</tr>
<tr>
<td>zearelenone</td>
<td>estrogenic</td>
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</table>

**Mycotoxin Effects on Insects**

For the most part, carcinogenicity is not of concern in insect studies due to the rarity of insect cancers. Thus, the reported effects of mycotoxins on insects include acute toxicity, reduction in growth rates, morphological, histological and reproductive changes. The two most extensively studied groups of mycotoxins are the aflatoxins and the trichothecenes. These compounds presumably act on insects in a manner similar to the way they act on mammals, by binding with DNA (aflatoxins) or by inhibiting protein synthesis (both). Insects fed these compounds may die, have reduced growth rates, or reduced fecundity. Aflatoxin B1 is reported to induce recessive lethal mutants in *Drosophila melanogaster* (6). The tremorgenic mycotoxins interact with the insect nervous system presumably as they do in vertebrates, by affecting γ-aminobutyric acid (GABA), glutamic, or other transmitters/receptor systems (5). Those that affect nitrogen regulation and manifest their effects on the mammalian kidney can also affect the corresponding structures in insects, the Malpighian tubules (7). In fact, virtually all mycotoxins have some effect on insects at naturally occurring concentrations (typically 25 ppm) (5). Thus, it is easy to understand why mycotoxins are considered as defenses against insects, as well as mammals. The implications of this concept are that all of the reactions and interrelationships known for insects and plant secondary metabolites can also be applied to insects and mycotoxins.
Insect "Resistance" to Mycotoxins

Insect resistance to mycotoxins may have evolved through continuous exposure and/or targeted feeding. Resistance to plant secondary metabolites and synthetic insecticides is sometimes thought to result from predisposed adaptations to (for example) plant secondary metabolites that have particular functional groups in common. The same situation is relevant for mycotoxins. For example, detoxifying enzyme systems that evolved in insects to dealkylate plant allelochemicals may be capable of dealkylating mycotoxins. Possibly, insect resistance to mycotoxins is ancestral to resistance to plant allelochemicals. Based on speculated times of origin, insects (8) and fungi (9) have been interacting for nearly 400 million years. This is approximately 275 million years longer than the period that insects have been interacting with flowering plants (based on a time of origin 125 million years ago - 10). Parallel evolutionary development of resistance to mycotoxins and plant allelochemicals by insects is also a possibility. Interestingly, one of the "oldest" insect groups, the cockroaches, is also one of the most resistant to aflatoxins (11). The aflatoxins themselves may represent the present day biosynthetic endpoint for a progression of precursors that show a decreasing toxicity to insects the further they are removed from aflatoxins (12).

Insects with similar host ranges have different sensitivities to mycotoxins even though they would not be expected to have adapted to mycotoxins due to targeted feeding. One example is Spodoptera frugiperda and Helicoverpa zea, both of which may feed on corn or other plants occasionally contaminated with mycotoxins. Based on sublethal effects at 0.25 ppm (13), H. zea is about 10-fold more sensitive to aflatoxin B₁ than S. frugiperda. In contrast, S. frugiperda is more sensitive than H. zea to the tremorgen roseotoxin B at 25 ppm in diets (100% vs. 38% mortality, respectively). The converse is true for the tremorgen penitrem A (20% vs. 80% reduction in growth rates, respectively, at 0.25 ppm in diets) (14). Griseofulvin, a mycotoxin that is also used pharmaceutically to treat fungal skin infections, is more toxic to H. zea than S. frugiperda (Dowd, P.F., unpublished data - see following discussion). On the other hand, most trichothecenes are of similar toxicity to both insects (5,15-17).

There is also variability in susceptibility of different strains of Drosophila melanogaster to aflatoxin B₁. At 400 ppb, all of the Crimea strain died, 24% of the Swedish-C strain reached adulthood and approximately 70% of the Hikone-R, Lausanne-S, and Oregon-R strains reached adulthood (18). These differences in susceptibility suggest that some sort of resistance mechanism(s) are present.

There are other examples where continuous exposure has apparently produced resistance to mycotoxins in insects. Stored product beetles appear relatively resistant to ochratoxin A and T-2 toxin (19) compared to stored product caterpillars (20). Possibly the beetles are less mobile than the moths and have experienced greater selection pressure when stored materials have become contaminated with mycotoxin-producing fungi.
There are also cases where targeted feeding on mycotoxin-contaminated materials has produced high levels of mycotoxin resistance. The different species of Drosophila that feed on mushrooms, potentially containing the toxin α-amanitin, are much less susceptible to α-amanitin than are unadapted, fruit-feeding species. Fruit-feeding Drosophila species D. melanogaster, D. pseudoobscura and D. immigrans were adversely affected by dietary concentrations of α-amanitin at 50 ppm or less, while the mushroom feeding D. recens, D. putrida and D. tripunctata were not affected (21). Similarly, the mushroom-feeding D. recens, D. falleni and D. phalerata were able to develop at least to pupae at α-amanitin concentrations of 50 ppm while the closely related but detritus-feeding D. quinaria, D. palustris, and D. subpalustris were not (22). However, one strain of D. melanogaster was also resistant to α-amanitin (21). The ability to feed on amanitin-containing mushrooms also appears to rid the flies of parasitic nematodes, which are adversely affected by the α-amanitin (22).

Large sclerotia (long term survival structures of fungi) of the ergot fungus Claviceps spp. contain a number of neuroactive compounds, and are fed on by phalacrid beetles (Coleoptera: Phalacridae) (23,24). Finally, sap beetles (Coleoptera: Nitidulidae) are reported to feed on and spread a variety of mycotoxigenic fungi (25). Both adults and larvae of a representative sap beetle, Carpophilus hemipterus are only slightly, if at all, affected by short term exposures to representative Aspergillus, Penicillium and Fusarium mycotoxins at 25 ppm, including aflatoxin B₁, sterigmatocystin, citrinin, cyclopiazonic acid, penicillic acid, diacetoxyscirpenol, deoxynivalenol, and T-2 toxin (5). Thus, there are a number of insects that are relatively resistant to mycotoxins. In this review, the resistance mechanisms present in D. melanogaster, S. frugiperda, H. zea and C. hemipterus will be emphasized.

Resistance Mechanisms

Although not specifically adapted for feeding on mycotoxin-containing materials, the strains of D. melanogaster resistant to aflatoxin B₁ and α-amanitin have been investigated in some detail. Unfortunately, initial attempts at increasing resistance levels in the aflatoxin-resistant D. melanogaster, by selection for 18-20 generations, yielded strains with less than a 3-fold increase (26). Isogenic lines of Oregon-R and Lausanne-S were approximately 3 x more susceptible to 1.0 ppm of dietary aflatoxin B₁ than nonisogenic ones (27). This information suggests that the strains have not attained "true resistance" whereby genes conferring resistance have assorted appropriately. Hybrids between resistant (Lausanne-S) and susceptible (Florida-9, Canton-S, Swedish-C) strains were intermediate in resistance, with 85% of the Lausanne-S, 30% of the hybrids, and none of the susceptible strains surviving 1050 ppb of aflatoxin B₁ in diets (28). The authors suggested that multiple genes were likely to be involved (28).
Chromosomal substitution studies initially indicated that autosomal genes on chromosomes 2 and 3 in the Lausanne-S strain control the resistance to aflatoxin B$_1$ (29). However, diallele analysis indicated that nuclear gene differences (chromosome X and 2) were responsible for differences in resistance, that some of these genes showed additive interactions, and that some of the gene expression (on chromosome 2) is regulated by cytoplasmic factors (30). As a result of these observations, the authors suggested that while quantitative/qualitative differences in P-450 xenobiotic-metabolizing enzymes (unspecific monooxygenases) may be partly involved, this did not explain the cytoplasmic-nuclear gene interactions (30). Unfortunately, the mechanisms of resistance to aflatoxin B$_1$ in these strains have not been determined.

The mechanism(s) for resistance to $\alpha$-amanitin for the C-4 strain of D. melanogaster has been determined, and involves an altered target site. The $\alpha$-amanitin appears to cause toxic effects by binding to RNA-polymerase and thereby inhibiting its function and subsequent protein synthesis. Jaenike et al. (21) found that compared to other Drosophila spp., including those resistant to $\alpha$-amanitin, the RNA-polymerase II of the resistant strain of D. melanogaster retained at least 50% of its activity at concentrations of 5 ppm of $\alpha$-amanitin in vitro, while that of the other Drosophila spp. was inhibited greater than 50% by $\alpha$-amanitin at 0.1 ppm. Mechanisms of $\alpha$-amanitin resistance in the mushroom-feeding species of Drosophila have not been determined.

Research conducted in our laboratory has concentrated on S. frugiperda and H. zea as representative insects that have differing susceptibilities to mycotoxins, and yet are not specifically adapted to feeding on mycotoxin-contaminated materials. As indicated before, these species have obvious differences in susceptibility to some, but not all, mycotoxins. In addition, they have been well studied for responses to, and metabolism of, plant allelochemicals and insecticides. A few "case studies" will now be discussed to indicate potential resistance mechanisms to mycotoxins present in unadapted insects, which might be considered cases of "predisposition". Although these studies are oriented towards activities of detoxifying enzymes, it is just as likely that other detoxifying mechanisms, such as sequestration or excretion, could also be involved.

When NADPH-associated oxidation assays were performed using midguts from H. zea and S. frugiperda, only two of over a dozen mycotoxins tested were significantly metabolized (Dowd, P.F., unpublished data). One of these, zearalenone, is a steroid-based compound that may be metabolized by enzymes associated with the regulation of ecdysone or other steroids in insects. The other, sterigmatocystin, is a biosynthetic precursor for aflatoxin B$_1$, and has moieties (e.g. double bonds, alkyl groups) that also may be activated or detoxified.

Aflatoxin B$_1$ is an important Aspergillus mycotoxin and was selected for more detailed studies. In vertebrates and microorganisms, aflatoxin B$_1$ can undergo activating (generation of a reactive epoxide at the 2,3 position) and detoxifying (O-demethylation, glutathione conjugation) reactions. As indicated earlier, S. frugiperda is less sensitive to aflatoxin
B<sub>1</sub> than H. zea. Thus, it was of interest to investigate potential detoxifying strategies to determine the cause of "resistance" of aflatoxin B<sub>1</sub> in S. frugiperda compared to H. zea.

To facilitate this investigation, a reverse-phase TLC method was developed. This method readily separated bound material (origin), the parent aflatoxin B<sub>1</sub> (Rf ca. 0.5), potentially activated compounds (lower Rf than aflatoxin B<sub>1</sub>) and detoxified metabolites, with the more polar (and presumably less toxic) metabolites having a higher Rf than aflatoxin B<sub>1</sub>. Different cofactors, such as glutathione and NADPH, were used to determine potential types of enzymes involved. The intense and characteristic blue fluorescence of aflatoxin B<sub>1</sub> and some of the other metabolites at the concentration used provided an immediate indication of metabolic strategies. Based on relative polarities and the limited number of standards available, H. zea is more likely to activate, and less likely to produce relatively polar, less toxic metabolites than is S. frugiperda (Figure 2).

Quantitation and identification of the metabolites is still in progress. Although NADPH enhanced production of some metabolites in H. zea, it is presently unknown whether the glutathione conjugates are formed from the activated, intermediate epoxide, as occurs in rats (31). Interestingly, the fungus can compensate for a predator's oxidative detoxification of aflatoxin B<sub>1</sub> by producing large quantities of kojic acid, which inhibits oxidative detoxification of other compounds (13,15). Whether kojic acid is selective for detoxifying vs. activating monoxygenases is presently unknown.

A representative Penicillium mycotoxin with differential toxicity to S. frugiperda and H. zea is griseofulvin. Again, different rates of detoxification and activation appear to explain the disparity in toxicity in these two insect species. However, photoactivation of this compound (Dowd, P.F., unpublished data) is a further complicating factor. Griseofulvin was metabolized rapidly in midguts of H. zea, and the metabolism was almost completely inhibited by piperonyl butoxide (Table II). In S. frugiperda, metabolism was about half the rate of that in H. zea, and piperonyl butoxide only inhibited metabolism by about 50%. However, in oral toxicity assays, piperonyl butoxide was more effective in synergizing the toxicity of griseofulvin in S. frugiperda than H. zea (where toxicity was antagonized).

Although a number of explanations are possible, apparently in H. zea some sort of monoxygenase-dependent activation occurs, while in S. frugiperda monoxygenase-dependent detoxification is more important. These metabolism profiles are consistent with the differences in toxicity.

As discussed earlier, the toxicity profiles for most trichothecenes are very similar for H. zea and S. frugiperda. Detoxifying enzymes can often be induced by plant allelochemicals or insecticides, which is presumably due to de novo protein synthesis (32). As foreign compounds, trichothecenes may potentially induce detoxifying enzymes in insects, but as protein synthesis inhibitors, trichothecenes could also depress these same activities. Interestingly, when H. zea and S. frugiperda were used to examine this relationship,
Figure 2. Aflatoxin B₁ and metabolites separated by thin-layer chromatography and visualized under long wave UV light. a. H. zea, b. S. frugiperda. Bluish spots indicated with "B" and greenish spots indicated with "G".
unspecific monoxygenase and glutathione transferase activities were sometimes induced by 250 or 25 ppm of deoxynivalenol, T-2 toxin, and diacetoxyscirpenol (16). Surprisingly (based on earlier work with esterases, which indicates levels of esterase induction are typically very low, e.g. 32), a new esterase (as indicated by gel electrophoresis and stain for 1-naphthyl acetate esterase) was also induced in both insects by these concentrations of trichothecenes (16). Based on indirect assays, such as using 1-naphthyl acetate as a competitive inhibitor, this esterase appeared to be responsible for hydrolyzing acetylated trichothecenes, including the model substrate monoacetoxyscirpenol (16). Thus, detoxifying enzyme systems in insects can also respond to mycotoxins by synthesizing greater levels of detoxifying enzymes. Although not yet examined, this capability may also be involved in insect resistance to mycotoxins.

Table II. Toxicity and Metabolism of Griseofulvin Alone and in Combination with Piperonyl Butoxide

<table>
<thead>
<tr>
<th></th>
<th>Oral toxicity (% Control weight)</th>
<th>Metabolism (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- piperonyl butoxide</td>
<td>+ piperonyl butoxide</td>
</tr>
<tr>
<td></td>
<td>griseofulvin</td>
<td>griseofulvin</td>
</tr>
<tr>
<td>S. frugiperda</td>
<td>100.0</td>
<td>79.7</td>
</tr>
<tr>
<td>+ griseofulvin</td>
<td>82.0</td>
<td>43.5</td>
</tr>
<tr>
<td>H. zea</td>
<td>100.0</td>
<td>74.9</td>
</tr>
<tr>
<td>+ griseofulvin</td>
<td>45.0</td>
<td>34.3</td>
</tr>
</tbody>
</table>

Metabolism is based on 3 hour incubations of 1 intact gut with 100 nmole of griseofulvin.

Insects Resistant to Mycotoxins

In spite of the number of examples given earlier where selected species of insects have demonstrated resistance to mycotoxins, relatively few studies have investigated the resistance mechanisms in insects that frequently or preferentially feed on materials contaminated with mycotoxins.
As mentioned earlier, the resistance mechanism of the mushroom-feeding Drosophila spp. to α-amanitin has not been determined. The resistance mechanism of the phalacrid beetles that feed on ergot is also unknown. However, symbionts in the cigarette beetle, Lasioderma serricorne (Coleoptera: Anobiidae), may contribute to this insect’s resistance to mycotoxins. The apparent detoxification of ochratoxin A by symbionts has been detected histochemically (33). Cultures of the symbiont can apparently utilize mycotoxins such as ochratoxin A, deoxynivalenol, citrinin, mycophenolic acid, and sterigmatocystin as carbon sources, also suggesting an ability to detoxify them (34). Studies on the ability of cultured symbionts to detoxify aflatoxin B₁ are in progress. However, metabolic studies in the presence and absence of symbionts in insects fed mycotoxins are needed to clarify this possibility.

 Sap beetles also are resistant to the effects of mycotoxins. The model trichothecene monacroctoxyscirpenol was primarily detoxified by hydrolysis at 8 to 10 fold the rate of H. zea and S. frugiperda (35). This information suggests that enhanced enzymatic detoxification contributes to trichothecene resistance in sap beetles. Studies with aflatoxin B₁ (Dowd, P.F., unpublished data) indicated NADPH-enhanced production of a metabolite. This metabolite had a slightly lower RF than aflatoxin B₁ by reversed-phase TLC, but quantitation is not yet complete. Possibly, altered target sites or simple excretion or sequestration are also involved in resistance.

Summary and Utility

The preceding discussion has indicated that, as is the case for insect resistance to plant allelochemicals, enzymatic detoxification contributes to mycotoxin resistance in insects, although altered target sites are also a possibility. Similar to the case for plant allelochemicals and insecticides, insect systems respond to dietary mycotoxins by synthesizing new or increased levels of detoxifying enzymes. Resistance to mycotoxins has allowed some insects to exploit substrates that are unavailable to other species. In some cases, insects resistant to mycotoxins may deliberately carry mycotoxin-producing fungi and inoculate materials so that the subsequent production of mycotoxins excludes competing insects (25).

Many questions remain to be answered, because there is only a limited amount of research that has been performed on mycotoxin resistance in insects. Mycotoxins (4,14), other fungal metabolites (5) or their derivatives are potentially a good source for novel insect control agents. By studying resistance mechanisms to fungal metabolites such as mycotoxins, it may be possible to evaluate the potential for resistance development as compared to plant-derived or other microbially-produced insecticides. Information gained from these studies may permit determination of whether mycotoxin resistance mechanisms are ancestral to others and help direct the design of new insecticides.

The contamination of foodstuffs and feedstuffs by mycotoxins continues to be a worldwide problem. Insects are probably the only group of organisms that have widespread resistance to mycotoxins.
Isolation and characterization of receptors and enzymes involved in detoxification may reveal mechanisms that can be applied to other situations. For example, incorporation of insect-derived genes for mycotoxin detoxification into appropriate microorganisms may allow for bioremediation of toxin-contaminated animal feeds or chemical feedstocks. Even the insects could be used as biocconversion mechanisms. In conclusion, the study of insect resistance mechanisms to mycotoxins may yield information applicable to solutions for a variety of problems associated with both insecticide resistance and mycotoxin contamination.

Literature Cited

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