TOXICITY OF MYCOTOXINS TO INSECTS AND UNDERLYING MOLECULAR AND BIOCHEMICAL MECHANISMS

BY

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DISSERTATION

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Abstract

Mycotoxins are synthesized by fungi and released as secondary metabolites toxic to many animal species. The structurally diverse mycotoxins can be carcinogenic, mutagenic, teratogenic, estrogenic, hemorrhagic, immunotoxic, nephrotoxic, hepatotoxic, dermatotoxic, and neurotoxic to humans and other mammals. To minimize the harmful effects of mycotoxins on humans, strict regulations have been established worldwide. Economic losses due to mycotoxin contamination are enormous and estimated at millions of dollars annually in the United States alone.

Insects inevitably encounter mycotoxins in nature when they feed on unharvested (mummy) fruits in orchard situations. Insects can be vectors of fungal spores and also facilitate fungal access to their host plants. Insects in turn may take advantage of fungi for protection against their natural enemies and for processing refractory plant constituents to increase their digestibility. This interaction is manifested in many crops as an association between insect damage and increase in mycotoxin contamination. Control of insects can thus reduce fungal toxin levels in crops. Understanding how insects deal with mycotoxins is critical in control of both insect pests and mycotoxin contamination.

In this study, three insects, including *Helicoverpa zea* (corn earworm), *Amyelois transitella* (navel orangeworm) and *Apis mellifera* (honey bee) varying in their exposure to mycotoxins were selected to study toxicity of mycotoxins to insects and to elucidate their underlying mechanisms of toxicity. To compare toxicity of mycotoxins to *H. zea* with that to *A. transitella*, I measured the developmental delay caused by aflatoxin B1 (AFB1), and found that the LC50 (defined as the concentration preventing 50% of newly hatched larvae from entering the second instar within 48 hr) for AFB1 is 100 times greater for *A. transitella* than that for *H. zea*. Similarly, *A. transitella* first instars display substantially higher tolerance of ochratoxin A (OTA) than do *H. zea*. Honey bees and their resource-rich nests are hosts to a wide range of saprophytic fungi, including species that produce mycotoxins. Bioassays showed that the honey bee workers can tolerate relatively high levels of aflatoxins (1-2.5μg/g AFB1) and ochratoxins (1μg/g OTA).
Cytochrome P450 monooxygenases (P450s) are critical in detoxification and activation of mycotoxins. In this study, I found that midgut homogenates isolated from *H. zea* larvae consuming diets supplemented with phytochemicals (coumarin and xanthotoxin) showed significant AFB1 disappearance and generated two metabolites, with the primary one identified as aflatoxin P1 (AFP1), an O-demethylated less toxic product of AFB1. Three P450 proteins from *H. zea* including CYP6B8, CYP6B27 and CYP321A1 were co-expressed with house fly reductase in insect cells and only the expressed CYP321A1 can metabolize AFB1, producing the same two metabolites as the midgut homogenates. RT-PCR gel blots indicated that the magnitude of CYP321A1 transcript induction by these chemicals is associated with the magnitude of increase in the metabolic activities of induced midgut enzymes (coumarin>xanthotoxin>indole 3-carbinol). These results indicate that induction of P450s, such as CYP321A1, plays an important role in reducing AFB1 toxicity to *H. zea*. Docking of AFB1 in the molecular models of CYP321A1 and CYP6B8 highlights differences in their proximal catalytic site volumes that allow only CYP321A1 to generate the AFP1 metabolite.

Enhancement of the toxicity of AFB1 by piperonyl butoxide, a P450 inhibitor, indicates a role for P450s in AFB1 detoxification in honey bees. Extracts of propolis, a complex mixture of plant-derived chemicals that contains many flavonoids and other phenolic compounds, similarly ameliorated aflatoxin toxicity and delayed the onset of mortality. Collectively, these results suggest that tolerance of AB1 by honey bees may be due to P450-mediated metabolic detoxification.

To understand molecular mechanisms underlying detoxification of mycotoxins by navel orangeworms, three full-length P450 cDNAs, including CYP6AB11, CYP321C1 and CYP6B44, were isolated from larval midguts using Rapid Amplification of cDNA Ends PCR. These P450s were co-expressed with house fly reductase and fruit fly cytochrome b5 in Sf9 insect cells infected with recombinant baculoviruses. Assays conducted with 16 compounds, including AFB1, showed that CYP6AB11 can efficiently metabolize imperatorin (0.88 pmol/min/pmol) and slowly metabolize PBO (0.11 pmol/min/pmol).
In view of all of results, it is clear that similar metabolic pathways of detoxification of mycotoxins similar to those found in mammals exist in insects. However, some insects, such as navel orangeworm, may have evolved in response to specializing on food containing mycotoxin-releasing fungi such as navel orangeworm. A potentially safe and sustainable approach for managing this serious fungus vector in orchards may be to use natural essential oil synergists. My results show that myristicin, a natural essential oil compound, synergized the toxicity of \( \alpha \)-cypermethrin, a pyrethroid insecticide, over time and slightly increased toxicity of a plant toxin, xanthotoxin, after seven days. Myristicin should be explored further as a field treatment to reduce survival of this pest species and to prevent aflatoxin contamination in orchard situations.
This thesis is dedicated to my families for their support and love.
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I. Interactions between insects and mycotoxins

Abstract

Mycotoxins are synthesized by fungi and released as secondary metabolites which are toxic to many animal species. The structurally diverse mycotoxins can be carcinogenic, mutagenic, teratogenic, estrogenic, hemorrhagic, immunotoxic, nephrotoxic, hepatotoxic, dermotoxic, and neurotoxic to humans and other mammals. Cytochrome P450 monooxygenases (P450s) are critical in detoxification and activation of these toxins, and differences in P450 activity lead to variable susceptibility among species. Insects inevitably encounter mycotoxins in nature when they feed on unharvested (mummy) fruits in orchard situations. These fruits are frequently colonized by fungi and the co-occurrence of herbivorous insects with fungi has affected their ecology and evolution. Insects can be vectors of fungal spores and also facilitate fungal access to their host plants. Insects in turn may take advantage of fungi for protection against their natural enemies and for processing refractory plant constituents to increase their digestibility.

With the frequency of encountering mycotoxins varying among species, insects have developed variable levels and mechanisms of tolerance. The determination of the metabolic pathways of mycotoxins in insects and identification of particular enzymes involved will shed light on understanding the factors that contribute to variation in tolerance among different insect species and strains. Determination of molecular and biochemical mechanisms of detoxification and activation of mycotoxins in insects will aid researchers in developing sustainable means for managing fungal vectors and will allow farmers to benefit by growing insect- or fungus- resistant crop strains or transgenic plants in the future.
1. Introduction

Mycotoxins are defined as the chemicals synthesized by fungi and released as toxic secondary metabolites. Most mycotoxins are of low molecular weight (<700), with highly variable structures. They are usually not involved in fungal growth and development. Their toxicity to animals in many cases depends on bioactivation by metabolic enzymes. The mycotoxin-releasing molds infect a broad range of plants and ingestion of mycotoxins leads to diseases and death for many mammals, poultry and fish (Wogan, 1975; Neal, 1995; Hussein and Brasel, 2001; Molyneux et al., 2007).

Insects encounter mycotoxins when they consume fungus-infected mummy fruits from their host plants. This co-occurrence of herbivorous insects with fungi has affected their ecology and evolution. Fungi can be pathogens of insects, with infection leading to symptoms such as reduction of feeding, delayed development, decreased mating success and even death (Roberts and St Leger, 2004; Scholte et al., 2004; Pedrini et al., 2007). Fungi can also be symbionts of insects with a mutualistic association leading to enhanced digestive efficiency and higher fitness (Brownlie and Johnson, 2009; Hartley and Gange, 2009). These two types of interactions arise from the generally fortuitous associations between insects and mycotoxin-releasing fungi. Insects, themselves, are not hosts for mycotoxin-releasing fungi and both the insect and the mycotoxin-releasing fungus may share the same plant host. Insects can serve as vectors of mycotoxin-releasing fungi and also facilitate fungal access to their plant hosts. Insects in turn may take advantage of fungi for protection against their natural enemies and for processing refractory plant constituents to increase their digestibility. Long-term trophic association may have promoted co-evolution between mycotoxin-releasing fungi and herbivorous insects attacking the same host plants.

To minimize the harmful effects of mycotoxins on humans, strict regulations have been established worldwide. Economic losses due to mycotoxin contamination are enormous and estimated at millions of dollars annually in the United States alone (Molyneux et al., 2007). Control of insects has been considered as an effective method to reduce mycotoxin levels in crops. To summarize the recent
research in interaction between insects and mycotoxins, I will discuss mycotoxins and their metabolism in animals, describe the association between insect damage and mycotoxin contamination in crops, and summarize studies in toxicity of mycotoxins to insects and their underlying resistance mechanisms.

2. Mycotoxins

Over 300 mycotoxins have been isolated and identified (Betian, 1984) but only a small number of mycotoxins known to cause serious diseases in humans and other mammals have been studied; these include aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, tremorgenic toxins, and ergotoxins (Steyn, 1995; Hussein and Brasel, 2001; Molyneux et al., 2007). Most of these toxins are released by several species of fungi. The most common mycotoxin-releasing fungi are *Aspergillus* species. The structurally diverse mycotoxins can be carcinogenic, mutagenic, teratogenic, estrogenic, hemorrhagic, immunotoxic, nephrotoxic, hepatotoxic, dermotoxic, and neurotoxic to vertebrates, including humans (Steyn, 1995). Mycotoxins can cause various chronic or acute diseases depending on their different modes of action. Aflatoxins were the first mycotoxins to be identified when, in 1960, an aflatoxin releasing-fungus, *Aspergillus flavus*, infected more than 100,000 young turkeys in England with high mortality after a few months. The disease was initially named “Turkey X disease” (Eaton and Gallagher, 1994; Neal, 1995; Hussein and Brasel, 2001) until mycotoxins were identified as the causative agent. Since then, toxicity, metabolism and impact of aflatoxins and other mycotoxins on humans and other animals have been widely studied.

In nature, there are four common aflatoxins, including aflatoxin B1, B2, G1, and G2. Among them, aflatoxin B1 (AFB1) and B2 (AFB2) are released mainly by *A. flavus* and *Aspergillus parasiticus* and aflatoxin G1 (AFG1) and G2 (AFG2) are released mainly by *A. parasiticus*. Aflatoxin B1 is most toxic among the four aflatoxins and also one of the main carcinogens responsible for human liver cancer (Eaton and Gallagher, 1994; Neal, 1995; Hussein and Brasel, 2001). The toxicity of AFB1 is typically induced by the metabolic system in liver or lung to form an unstable but extremely toxic product, AFB1-epoxide (AFBO), which can cause DNA
mutations. Aflatoxins cause acute and chronic toxicity to vertebrates by directly damaging tissues, altering vital gene expression and interfering with cell apoptosis (Eaton and Gallagher, 1994; McLean and Dutton, 1995; Neal, 1995; Guengerich et. al, 1998). Studies have also shown that co-occurrence of Hepatitis B virus (HBV) with aflatoxin contamination in foods can increase the risk of hepatocellular carcinoma. The International Agency for Research on Cancer (IARC) from the World Health Organization has classified aflatoxins as Class I toxins (Henry et al., 1999). Aflatoxin B1 toxicity varies among species, with the turkey among the most susceptible species (Eaton and Gallagher, 1994) while some rodent species are relatively tolerant (Wong and Hsieh, 1980).

Aflatoxins are generally not acutely toxic to humans but their chronic effects, especially their carcinogenic effects, make them among the most dangerous natural chemicals. Aflatoxin-releasing fungi can infest most commodities and feeds. Complete elimination of aflatoxin contamination is not an achievable goal because contamination can occur in every stage of food production, including growth, harvest, processing, delivery and storage (Park and Troxell, 2002; Cleveland et al., 2003). In the United States, the Food and Drug Administration limits total aflatoxin levels to 20 ppb in all foods and 0.5 ppb AFM1 in milk (Food and Drug Administration, 1996). The European Union sets much more stringent regulations, with upper contamination limits set at 4 ppb-8 ppb in common foods and 0.05 ppb AFM1 in milk (van Egmond et al., 2007). In most developed countries, aflatoxins are primarily an economic and not a health problem. However, in some developing countries, especially in Africa and Asia, aflatoxins may cause death with long time exposure. The occurrence rate of liver cancer is significantly higher in developing nations and is associated with the combined effects of high frequencies of hepatitis B viral infection and daily diets containing high levels of aflatoxins in these areas (Henry et al., 1999; Suriawinata and Xu, 2004; Williams et al., 2004).

In addition to aflatoxins, *Aspergillus* species, as well as some *Penicillium* species, produce another class of mycotoxins, the ochratoxins. Ochratoxin A (OTA), the most widespread and toxic compound in this group, is known for its nephrotoxic
effects in poultry and is considered a potential human carcinogen (Dirheimer and Creppy, 1991; Marin-Kuan et al., 2008). The sesquiterpene trichotheccenes constitute another group of mycotoxins, produced by various fungi, including species in the genera *Fusarium*, *Myrothecium*, *Trichoderma*, *Trichothecium*, *Cephalosporium*, *Verticimonosporium*, and *Stachybotrys*. T-2 toxin, the most toxic trichotheccene, is produced mainly by *Fusarium* species. It owes its toxicity to vertebrates to its ability to inhibit DNA and RNA synthesis, causing cytotoxicity, cell damage and immune system changes (Steyen, 1995; Sokolović et al., 2008). Zearalenone, yet another mycotoxin, is a potent phytoestrogenic compound synthesized by several *Fusarium* species that can cause infertility, abortion or other reproductive problems. Fumonisins (B1 and B2) are potential carcinogens and metabolites of *F. proliferatum* and *F. verticillioides*, which frequently infect maize, wheat and other grain crops. Fumonisin B1 can cause esophageal cancer and neural tube defects in humans and also cause equine leukoencephalomalacia and porcine pulmonary edema (Steyen, 1995; Stockmann-Juvala and Savolainen, 2008). Most tremorgenic toxins (Selala et al., 1989) are produced by molds from the genera *Penicillium* and *Aspergillus* and can damage the central nervous system (CNS). Ergotoxines are mixtures of alkaloids identified from the ergot fungus *Claviceps purpurea* that can produce intensive generalized vasoconstriction of blood vessels in human (Bangh et al., 2005).

3. Metabolism of aflatoxins

Although the metabolic breakdown of mycotoxins has been studied in a wide range of animals, only that for aflatoxins has been elucidated. Among enzymes responsible for the biotransformation of aflatoxins across species are cytochrome P450 monooxygenases (P450s), a ubiquitous superfamily of heme proteins that enzymatically use NADPH and or/NADH to reductively split molecular dioxygen, binding one oxygen to the substrate and forming water with the other oxygen atom (Feyereisen, 1999). The multiplicity and diversity of P450s allow them to metabolize a wide range of chemicals, including steroid hormones, fatty acids, phytochemicals, pollutants and carcinogens (Feyereisen, 1999; Stegeman and Livingstone, 1998; Shuler and Werck-Reichhart, 2003; Li et al., 2007). Mammalian P450s have long
been associated with the biotransformation of aflatoxins (Fig. 1). Oxidation of AFB1 leads to formation of multiple polar metabolites, including aflatoxin M1 (AFM1), aflatoxin Q1 (AFQ1), aflatoxin B2a (AFB2a) and aflatoxin P1 (AFP1). Aflatoxin M1, Q1 and B2a are products of hydroxylation of AFB1 and display lower acute toxicity and mutagenicity than AFB1 (Sinnhuber et al., 1974; Hsieh et al., 1984; Raney et al., 1992). Aflatoxin P1, an O-demethylation product of AFB1, is the main metabolite in urine of rats, mice and monkeys exposed to dietary AFB1 (Wong and Hsieh, 1980).

While P450s generally metabolize xenobiotics into less toxic products, occasionally they can bioactivate certain compounds. Aflatoxin B1 is bioactivated by P450s to AFBO, which can interact with DNA to form guanyl N7 adducts leading to inhibition of DNA synthesis or generation of point mutations that activate protooncogenes and inactivate cancer suppressive genes. Activated products can also bind with proteins to directly affect the activity of functional enzymes (Eaton and Gallagher, 1994; McLean and Dutton, 1995; Guengerich et al., 1998). Phase II detoxification enzymes including glutathione S-transferases (GSTs), glucoside transferase and cytochrome reductase are also important in the detoxification of aflatoxins (Fig. 1). GST-mediated conjugation of AFBO with reduced GSH is a major pathway to detoxify AFB1 in many mammals. GST acts by conjugating glutathione (GSH) to electrophilic substrates to generate more hydrophilic and more excretable metabolites (Hayes et al., 2005). In rodents, susceptibility to AFB1 is inversely associated with GST activity in liver toward AFBO (Lotlikar et al., 1984; Monroe and Eaton; 1988; Buetler et al., 1992). Aflatoxin B1 can be converted to aflatoxicol (AFL) by aflatoxin B1 aldehyde reductase (AFAR) and converted back to AFB1 by a dehydrogenase (Eaton and Gallagher, 1994).

4. Association between insect damage and mycotoxin contamination in crops

Insect damage has long been associated with increased mycotoxin contamination in crops. Fruits with enclosed seeds are particularly suitable habitats for mycotoxin-producing fungi following herbivory by such insects as Helicoverpa zea, the corn earworm, Pectinophora gossypiella, the pink bollworm and Amyelois transitella, the navel orangeworm (Widerstrom, 1979). In addition, some other
insects such as *Elasmopalpus lignosellus* (the cornstalk borer), *Diabrotica undecimpunctata howardi* (the corn rootworm), *Microtermes spp* (termite), and other non-insect animals such as mites and nematodes also contribute to *Aspergillus* infection in plants (Widstrom, 1979).

*H. zea* (Lepidoptera: Noctuidae), the corn earworm, is a significant facilitator of mycotoxin contamination of maize. It has a wide range of host plants and is a serious pest in North America (Neunzig, 1963; Archer *et al.*, 1994; Capinera, 2001). Of over 100 host plants, many, such as corn and cotton, are frequently infected by *Aspergillus* fungi. In the case of maize, larvae eat corn silk initially, and then damage kernels. Wounds can facilitate fungal access into the ear (Widstrom, 1979). Many lines of evidence indicate a causative association between insect infestation and mycotoxin contamination. Higher levels of aflatoxin are detected in corn damaged by insects than in undamaged corn (Anderson *et al.*, 1975; Lillehoj *et al.*, 1976); kernels with thick pericarps typically have lower levels of aflatoxins (Calvert *et al.*, 1978); and mechanically damaged ears are more likely to be infected by fungi (Lillehoj *et al.*, 1979).

Effective insect control is one way to reduce crop contamination by mycotoxins. Control of *H. zea* with insecticide application led to reductions in aflatoxin contamination in corn (Widstrom *et al.*, 1976). Hostplant resistance to insects is regarded as an economic way to reduce aflatoxin contamination. The use of transgenic Bt crops resistant to insects reduces both the direct damage by insect feeding and aflatoxin contamination of crops (Dowd, 2001; Buntin *et al.*, 2004; Wu *et al.*, 2006). Application of transgenic Bt crops prevents attacks from *H. zea* and also has decreased aflatoxin contamination (Dowd, 2001; Buntin *et al.*, 2004; Wu, 2006). Tex6, a maize inbred strain with resistance to both insect damage and *Aspergillus*, is a particularly promising tool for managing mycotoxin contamination (Dowd and White, 2002).

5. Toxicity of mycotoxins to insects

Mycotoxins can cause toxicity to insects, including insecticidal effects and developmental delay (Zeng *et al.*, 2006). There is variable sensitivity of insects to
aflatoxin toxicity, initially examined in the fruit fly *Drosophila melanogaster*. The fruit fly is frequently exposed to mummy fruits such as citrus fruits infected with *Aspergillus* fungi (Alderman and Marth, 1976). Ingestion of experimental medium to which a high concentration of AFB1, 10 ppm, was added caused death of all newly hatched first instar larvae in 8 days (Kirk et al., 1971). Other effects of AFB1 on *D. melanogaster* including egg-to-adult viability, fertility, and crossing over have been observed at a lower concentration, 674 ppb, which exceeds the LD$_{50}$ (Chinnici et al., 1976). Different wild strains of *D. melanogaster* also showed variable sensitivities to aflatoxins. The strain, Crimea, has a lower egg-to-adult viability than other wild strains when fed with 0.4 ppm AFB1 continuously (Llewellyn and Chinnici, 1978) and the hybrids of intercrossing of the Lausanne-S, a resistant strain, with Crimea strain showed relatively higher resistance to AFB1 measured with egg-to-adult viability and developmental time, and pupal case and adult body length (Chinnici et al., 1979). Strain variation suggests that AFB1 toxicity is genetically controlled and may be regulated by several genes conferring resistance. More interestingly, a sensitive strain, Florida-9, was more sensitive to AFG1 than AFB1, in contrast with the general pattern that AFB1 is more toxic than other aflatoxins in vertebrates; its lower sensitivity may be explained by its lower activity of activation of AFB1 (Gunst et al., 1982).

Aflatoxins also have been tested for their toxicity to larval lepidopterans. Toxicity of AFB1 was detected in newly hatched larvae of *Heliothis virescens*; 10 µg/g AFB1 can cause 100% mortality in 8 days (Gudauskas et al., 1967). *Aspergillus*-infected diet can cause mortality or growth delay to *Bombyx mori*, the silkworm (Ohtomo et al., 1975). Zeng et al. (2006) described toxicity of AFB1 to *H. zea* larvae at different larval stages. For newly hatched first instar larvae, 20 ng/g AFB1 caused 50% mortality while a higher concentration, 200 ng/g AFB1, caused 100% mortality. Pupal weight of the larvae fed with 1 ng/g AFB1 in diet was significantly decreased compared with control insects. Tolerance of AFB1 increases with larval stages. The third instar larvae can tolerate 20 ng/g AFB1 with only moderate reduction of pupal weight while 200 ng/g AFB1 significantly affected mortality, pupation rate and pupal
weight. For the fifth instar larvae, the concentration of 200 ng/g only delayed development without affecting mortality, pupation rate, or pupal weight, whereas 1 µg/g AFB1 reduced pupal weight by 29.3% and pupation rate by more than one-third. Malformation of pupae was found after the fifth instar larvae were exposed to 1µg/g AFB1 and the pupae failed to develop (Zeng et al., 2006).

6. Detoxification mechanism of mycotoxins by insects

Depending on the susceptibility of plants to *Aspergillus* infection, insects feeding on these plants encounter mycotoxins with variable frequency. How insects cope with mycotoxins has rarely been investigated. Insects have developed various mechanisms to deal with plant toxins (Ma et al., 1994; Hung et al., 1997; Li et al., 2004; Mao et al., 2006) and insecticides (Feyereisen, 1999; Sasabe et al., 2004; Li et al., 2007), with the most important mechanism being metabolic detoxification that generally involves two phases. In phase I, a functional group is usually added to the parent compound which is then conjugated to very water-soluble moieties such as glucuronic acid, sulfate, glutathione and others. Through these two phases, a lipophilic toxin is generally transformed into a more hydrophilic compound and excreted.

Up to twelve metabolites were observed in metabolism reactions carried out *in vitro* with tested tissue of different strains of *D. melanogaster* and the metabolites were identified as AFM1, AFB2a and AFL, with AFL as the primary metabolite among them (Foerster and Wurgler, 1984). Lee and Campbell (2000) found three metabolites of AFB1 produced by larvae of the navel orangeworm, including chiefly AFL and minor amounts of AFB2a and AFM1. Zeng et al. (2006) has demonstrated that P450s might be involved in both detoxification and bioactivation in *H. zea*. With addition of piperonyl butoxide (PBO), a general and effective inhibitor of P450s in many different taxa, to diet containing 1µg/g concentration of AFB1, toxicity of AFB1 to fourth instar larvae of *H. zea* was significantly decreased. Pupation rate significantly increased from 0% for larvae feeding on diet containing AFB1 alone to 71.7% for larvae feeding on diet containing both AFB1 and PBO. The fact that inhibition of P450 activity significantly decreases the toxicity of AFB1 indicates that
AFB1 is bioactivated to more toxic derivatives via one or more P450s in *H. zea*. On the other hand, fifth instar larvae exposed to both AFB1 and phenobarbital, a P450 inducer, significantly increased pupal weight compared with larvae feeding on the diet containing AFB1 alone. This finding suggests that induced P450s are responsible for the decrease in toxicity (Zeng et al., 2006). *Saccharomyces cerevisiae* transformed with fruit fly CYP6A2 cDNA and human NADPH-cytochrome P450 reductase has shown induced gene conversion when treated with AFB1, indicating that AFB1 might be activated to release its genetic toxicity (Saner et al., 1996). Similar metabolic pathways of detoxification and activity of aflatoxins in mammals may also exist in insects. Determination of molecular and biochemical mechanisms of detoxification and activation of mycotoxins in insects will aid researchers in developing sustainable means for managing fungal vectors and will allow farmers to benefit by growing insect- or fungus-resistant crop strains or transgenic plants in the future.

7. Summary

Mycotoxins are the ecological consequence of fungal adaptation to environmental challenges. Plants susceptible to fungus infection may produce mummy fruits with high concentrations of mycotoxins. Insects can greatly increase the infection rate by providing access routes for fungi and increase mycotoxin accumulation in these fruits. Co-evolution may have occurred in specific interactions between mycotoxin-releasing fungi and insects. Other than basic research, studies of interactions between insects and mycotoxins can contribute to agricultural applications. With concerns about health problem and economic losses due to mycotoxin contamination, people are looking for ways to prevent fungal infection in crops and to reduce mycotoxin levels in contaminated foods (Mishra and Das, 2003); understanding the interactions between insect damage and mycotoxin contamination can shed light on ways in which host plant resistance or transgenic plants can be employed to resolve both problems. Determination of the molecular and biochemical mechanisms of detoxification and activation systems of mycotoxins in insects will help researchers to understand how to control these fungal vectors and allow farmers
to gain benefits by growing insect- or mycotoxin-resistant crop strains or transgenic plants in the future.

References


Fig. 1 Detoxification and bioactivation of aflatoxin B1. (Eaton and Gallagher, 1994; Lee and Cambell BC, 2000)
II Comparative toxicity of mycotoxins to navel orangeworm (*Amyelois transitella*) and corn earworm (*Helicoverpa zea*)

Abstract

Mycotoxins, such as aflatoxins and ochratoxins, are widely distributed in nature and are frequently problematic crop contaminants causing millions of dollars of annual losses in the United States. Insect infestations of crop plants significantly exacerbate mycotoxin contamination. Damage to a variety of nut species by *Amyelois transitella* Walker (navel orangeworm, NOW) is associated with infection by *Aspergillus* species and concomitant production of aflatoxins and ochratoxins. Resistance to aflatoxins in this lepidopteran is compared here with the levels of resistance in *Helicoverpa zea* (corn earworm, CEW), another lepidopteran that routinely encounters aflatoxins in its diet, albeit at lower levels. Measured as the developmental delay caused by aflatoxin B1, it is apparent that the LC50 (defined as the concentration preventing 50% of newly hatched larvae from entering the second instar within 48 hr) for AFB1 is 100 times greater for *A. transitella* than for *H. zea*. Similarly, *A. transitella* first instars display substantially higher tolerance of ochratoxin A, another mycotoxin contaminant produced by *Aspergillus* species, than do *H. zea*. Our studies indicate that *A. transitella*, although a hostplant generalist, may well be highly specialized for mycotoxin detoxification.
1. Introduction

The navel orangeworm *Amyelois transitella* (Walker) (NOW: Lepidoptera: Pyralidae) is a serious crop pest that attacks almonds, pistachio, walnuts, peanuts and figs, causing millions of dollars of agricultural damage in the United States (Burks and Brandl, 2004; Campbell et al., 2003; Connell, 2001). Damage results from consumption of the nutmeat and contamination of the nut with webbing and frass. In addition to causing such direct losses, the feeding damage caused by NOW predisposes almonds to infection by *Aspergillus* spp., which produce toxic aflatoxins and ochratoxins. Particularly susceptible are soft-shelled cultivars with an extended split hull period (Schade et al., 1975; Schatzki and Ong, 2000, 2001). The occurrence of fungal contamination in pistachio, almond, peanut and figs is also closely associated with the extent of NOW damage (Campbell et al., 2003; Schatzki and Ong, 2000, 2001; Widstrom, 1979). Aflatoxin contamination has tremendous trade implications inasmuch as export to Europe is restricted based on minimum levels (Otsuki et al., 2001).

Aflatoxins are synthesized by *A. flavus* and *A. parasiticus*, with aflatoxins B1, B2, G1 and G2 most commonly produced (Cleveland et al., 2003). Of these, aflatoxin B1 (AFB1) causes a number of serious health problems in humans including acute and chronic toxicity to humans and mammals by directly damaging tissues, mutating important genes, inducing and inhibiting other vital gene expression and interfering with cell apoptosis (Eaton and Gallagher, 1994; Guengerich et. al, 1998; McLean and Dutton, 1995; Neal, 1995). Economic losses due to aflatoxin contamination are enormous and estimated at millions of dollars in the US alone each year. To minimize the harmful effects of aflatoxins, strict regulations have been established in the US. While crops and finished food products containing aflatoxins above 20 ppb are not allowed for human consumption, the limitation is set at 5ppb for milk (Cleveland et al., 2003; Williams et al., 2004). Ochratoxins are produced primarily by two other fungi, *A. ochraceus* and *Penicillium verrucosum*. Ochratoxin A (OTA) is capable of interfering with immune system function (Neal, 1995) and may also be carcinogenic. Because of these properties, commodities contaminated
with high concentrations of mycotoxins (e.g., 20 ppb for aflatoxins) are blocked from sale both in the U.S. and overseas (Bayman and Baker 2006; Park and Troxell, 2002). Without appropriate monitoring measures, mycotoxin-contaminated food has had serious impacts on human health in many areas, especially in developing countries in Asia and Africa (Williams et al., 2004).

The level of tolerance to mycotoxins in insects varies from several ppb to several ppm and is both species- and sex-specific (Chinnici et al., 1976; Chinnici and Llewellyn, 1979; Gudauskas et al., 1967; Kirk et al., 1971; Llewellyn and Chinnici 1978; ; Zeng et al., 2006). Larvae of *Drosophila melanogaster*, which feed naturally on fermenting fruits, exhibit significantly altered development on diets with low concentrations of AFB1 (less than 1 ppm) and on higher concentrations will die (Chinnici et al., 1976; Chinnici and Llewellyn, 1979; Kirk et al., 1971; Llewellyn and Chinnici 1978. The corn earworm, *Helicoverpa zea* (CEW), another species that encounters mycotoxin-releasing fungi in damaged plants (Archer and Bynum, 1994; Widstrom et al., 1976; Widstrom, 1979), is also tolerant of aflatoxins to some degree. Low concentrations of AFB1 in the diet of first instars (less than 100 ng/g) can affect development, causing delayed pupation and reduced pupal weight; higher concentrations of AFB1 (200 ng/g) are lethal (Zeng et al., 2006).

The toxicity of aflatoxins to insects depends on their metabolism as mediated by cytochrome P450 monooxygenases (P450s, Phase I detoxification enzymes) into bioactive products. One of the most toxic of these is the AFB1-exo-epoxide, which is derived from AFB1; this bioactivated metabolite of AFB1 exo-8,9-epoxide (AFBO) can bind directly to proteins and DNA and cause cell death (Eaton and Gallagher, 1994; Guengerich et al., 1998; McLean and Dutton, 1995; Suriawinata and Xu, 2004). Glutathione-S-transferases (GSTs, Phase II detoxification enzymes) conjugate glutathione to the lipophilic products of P450-mediated metabolism to form water-soluble metabolites that are more readily excreted (Hayes et al., 2005). In rodent livers, susceptibility to AFB1 is inversely associated with GST activity toward AFBO (Buetler et al., 1992; Lotlikar et al., 1984; Monroe and Eaton, 1988). Another biotransformation of AFB1 is its direct reduction by
NADPH-dependent aflatoxin B1 aldehyde reductases (AFAR) to form aflatoxicol (AFL), but this product is rapidly converted back to AFB1 by dehydrogenases (Eaton and Gallagher, 1994). Alternative biotransformation pathways that generate detoxified metabolites, such as aflatoxicol, aflatoxin M1 and aflatoxin B2a, and do not generate bioactivated epoxidized metabolites also exist in NOW and contribute to the high aflatoxin tolerance of this species (Lee and Campbell, 2000).

Characterization of similar biotransformation pathways in CEW indicate that at least one P450 in this species, specifically CYP321A1, is capable of converting AFB1 into polar metabolites, with the demethylated product aflatoxin P1 (AFP1) as the principal metabolite (Niu et al., 2008).

Given the existence of alternative biotransformation pathways in both NOW and CEW, we initiated a series of studies to compare toxicity and tolerance of mycotoxins in these two species. Because NOW is regularly associated with fungus-contaminated hostplant tissue, we hypothesized that its level of tolerance to both aflatoxins and ochratoxins should exceed that of CEW, which only occasionally encounters fungal contamination inasmuch as it feeds on fruits before they fall to the ground.

2 Methods

2.1 Chemicals

Piperonyl butoxide (PBO) was purchased from Tokyo Kassie Kogyo (Tokyo, Japan). Aflatoxins B1, G1 and M1, and ochratoxin A were purchased from Sigma Chemicals (St. Louis, MO). Analytical grade dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific (Pittsburgh, PA). All chemicals were dissolved in DMSO to make stock solutions and were stored at -20°C.

2.2 Insects

A laboratory colony of *H. zea* originating from specimens collected in Champaign County, IL and a colony of *A. transitella* originating on almond trees in Fresno, CA, which have been maintained in culture for the past 25 years by the Commodity Protection and Quality Unit, USDA/ARS, Parlier, CA, were kept in an insectary at UIUC at 28 ± 4°C with 16-hr light/8-hr dark cycles. *H. zea* larvae were
reared individually from egg hatch in 5 oz (142 g) plastic cups containing approximately 5 g control or supplemented diet. *A. transitella* larvae were mass-reared until pupation in 500 ml plastic containers containing 200 g of wheat bran diet (Tebbetts et al. 1978).

2.2 Bioassays

Stock solutions of AFB1 or OTA were prepared in DMSO and could be stored in −20 °C for two months. To make the diets containing 1, 5, 10, 20, 50 and 100 µg/g of AFB1, 100 µl or 500 µl of 1 mg/ml of AFB1 stock solution were incorporated into 100 g of the diet to make 1 µg/g or 5 µg/g of AFB1 diet in 0.5% DMSO; 50, 100, 250 and 500 µl of 20 mg/ml of AFB1 stock solution were added to 100 g of the diet to make 10, 20, 50 and 100 µg/g of AFB1 diets in 0.5% DMSO. For low concentrations of AFB1 diets containing 20, 40, 60, 80, 100, 120, 140 ng/g of the AFB1 diets, 20, 40, 60, 80, 100,120, 140 µl of 100 µg/ml AFB1 stock solution were added into 100 g of the diet in 0.2% DMSO. To prepare OTA diets, 50 or 250 µl of 1 mg/ml OTA stock solution were incorporated into 100 g of the diet in 0.25% DMSO to make 1 or 5 µg/g of the OTA diet; 50, 100, 250 µl of 20 mg/ml OTA were added into 100 g of the diet in 0.25% DMSO to make 10, 20, 50 µg/g of the OTA diet. Pilot experiments determined that concentrations of DMSO at levels as high as 0.5% of the diet do not affect development or survivorship in either NOW or CEW.

To assess the toxicity of AFB1 to NOW, six concentrations of AFB1 (1, 5, 10, 20, 50 and 100 µg/g) diets in 0.5% DMSO solution were prepared and twenty neonates (i.e., newly hatched first instars) were placed individually into 142-g plastic cups containing 0.5 g unamended diet or supplemented diet prepared with a final concentration of 0.5% DMSO. Two higher concentrations of AFB1 (50 and 100 µg/g) diets were fed to 20 ultimate (fifth) instars with the 0.5% DMSO as control. Observations of these larvae were made until pupation and pupal weights were measured within 48 hr after the final larval molt. Each set of bioassays was replicated three times.

The toxicity of OTA to NOW larvae was tested as for AFB1 with diets containing 1, 5, 10, 20 or 50 µg/g OTA at a final concentration in 0.25% DMSO;
twenty first instars in each group were treated. The proportions of dead larvae on the 12th day were recorded and each larva on the 14th day was weighed. The pupation rate was measured within 48 hours of pupation. To test the toxicity of OTA to CEW, first instars were exposed to the diets at concentrations of 0.25% DMSO, 1 or 5 µg/g OTA and control diet. The survivorship of larvae at 12 days post-hatch on diets and frequency of fifth instars at 12 days post-hatch were recorded.

To compare the effects of AFB1 on the development of NOW and CEW, first instar of CEW larvae were fed with artificial diets containing 0.2% DMSO or 10, 20, 40, 60, 80, 100, 120 or 140 ng/g AFB1 while first instar A. transitella were fed with artificial diets containing 0.2% DMSO, 1, 5, or 10 µg/g AFB1. For each species, the numbers of larvae reaching second instar were recorded within 48 hr.

2.3 Midgut protein preparations

To obtain sufficient midgut tissue from larvae, guts were dissected from at least 100 ultimate instars that had molted within the previous 12 hr. Midgut dissections were performed in 0.1 M phosphate buffer and on ice and then the midguts were frozen in liquid nitrogen and stored at -80°C until use. For each midgut preparation of ultimate instars, approximately 40 frozen midguts were ground in a mortar in the presence of liquid nitrogen, transferred into a 1.5 ml Eppendorf tube, suspended in 1.5 ml 0.1 M phosphate buffer (pH 7.4), 1 mM EDTA, 0.5 mM PMSF, 0.1 mM DTT, 20% glycerol and then gently shaken for 5 sec. These midgut homogenates were centrifuged for 10 min at 10,000 rpm in an Eppendorf centrifuge at 4°C and the cleared supernatant was transferred into a new 1.5 ml Eppendorf tube and either used immediately or frozen in liquid nitrogen and stored at -80°C for up to six months. Protein concentrations for these cleared lysates were determined using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

2.4 In vitro metabolism assays

For all assays, the frozen cleared lysates were taken from the -80°C freezer and thawed on ice for approximately 30 min before the metabolism assays were initiated. AFB1 and OTA metabolism assays were carried out as described previously in Lee and Campbell (2000) with only slight modifications. Stock solutions of AFB1
or OTA at concentrations of 10 µg/g were prepared in DMSO. Each 250-µl reaction contained 1µl AFB1 or OTA stock (10 µg/µl), 5 mM reduced glutathione, 1 mg/g protein, 0.1 mmol/L sodium phosphate buffer (pH 7.4) and 1 mM EDTA and was initiated with the addition of 50 µl of 3 mM NADPH. After a 60-min incubation at 30°C with shaking, 1 ml of ice-cold methanol containing 10 µM AFG1 (internal standard) was added to each reaction to terminate it. The reactions were then centrifuged at 10,000xg for 10 min in an Eppendorf centrifuge and each supernatant was transferred into a new 1.5 ml Eppendorf tube for analysis by high-pressure liquid chromatography (HPLC). All reactions were performed at least twice in triplicate. To trap the AFB1-exo-epoxide metabolite, mouse liver cytosol (10 µl, 50 mg/ml) was added into reactions containing NOW crude proteins. Reactions were stopped after incubation at 30°C for 1 hr and 10 µl of each supernatant were analyzed by HPLC.

AFB1 and its metabolites were separated on a reverse phase Supercosil LC-18 column (250 mm × 4.6 mm) on an HPLC system equipped with a photodiode array UV detector (Waters Novapak). Separation of AFB1 and products was achieved with water/methanol/acetonitrile (60/20/20) at a flow rate of 1 ml/min and monitoring at 362 nm. Under these conditions, AFG1 was eluted at 10.6 min and AFB1 was eluted at 14.6 min. Separation of OTA was achieved with acetic acid/methanol/acetonitrile (1/49/49) at a flow rate of 1 ml and monitoring at 333 nm; under these conditions, OTA was eluted at 10.4 min.

2.5 Statistics

All data were analyzed by one-way ANOVA analysis with a post-hoc Tukey test at a significance level of p < 0.05.

3. Results

3.1 Toxicity of aflatoxin B1 and ochratoxin A to NOW larvae

*A. transitella* larvae tolerated very high levels of AFB1 in their diet, with 1, 5, 10 and 20 µg/g AFB1 having no apparent toxic effects on first instars (Table 1). On the diets containing 10 µg/g and 20 µg/g AFB1, pupation rates were 77% and 78%, respectively, which did not differ significantly from pupation rates on control DMSO diet, and there was no significant difference in pupal weights between diets
containing 10 µg/g and 20 µg/g AFB1 and control diets. On the diet containing 50 µg/g AFB1, larval development was delayed and only 55% of the larvae successfully pupated, a rate significantly lower than the rate on the control diet (p < 0.05). At the extremely high AFB1 concentration of 100 µg/g, development of first instar *A. transitella* was severely inhibited, although 30% of the treated larvae survived for at least 3 weeks. Exposure of later stage larvae to diets containing varying concentrations of AFB1 showed that their tolerance to AFB1 increased with developmental stage; ultimate instars tolerated 100 µg/g AFB1 with no significant decreases in pupation rate compared to control larvae fed diets containing 0.5% DMSO (Table 2).

Similarly, *A. transitella* first instars also displayed great tolerance to OTA, with concentrations less than 10 µg/g having no detectable toxic effects (Table 3). At higher OTA concentrations (20 µg/g), there were no significant differences in mortality rates and larval weight after 14 days. Compared with control larvae fed diets containing 0.25% DMSO, *A. transitella* fed with the highest concentration of OTA (50 µg/g) experienced slight toxic effects on development, as evidenced by lower larval weights; larval weights after 14 days were significantly lower than those of larvae fed control diet (p = 0.047). The highest concentration of OTA (50 µg/g) did not significantly lower the pupation rate (Table 3).

### 3.2 Comparison of the toxicity of OTA and AFB1 to CEW and NOW

To compare the toxicity of AFB1 in *A. transitella* and *H. zea*, the effects of AFB1 on first instars of these two species were measured as described. The LC50 (defined as the concentration preventing 50% of newly hatched larvae from molting to second instar within 48 hr) for AFB1 is 100 times greater for *A. transitella* than for *H. zea* (Fig. 1). After 48 hr, 60 ng/g AFB1 allowed 50% of first instar *H. zea* to develop into second instars while 10 µg/g AFB1 allowed 80% of first instar *A. transitella* to develop to second instars, a greater than 100-fold difference in tolerance.

Comparisons of the toxicity of OTA to *A. transitella* and *H. zea* also revealed considerably greater resistance to OTA in *A. transitella*. Development of first instar *A.
transitella was not affected by concentrations as high as 1 and 5 µg/g OTA (table 3). These concentrations significantly inhibited the development of first instar H. zea compared with control larvae, with 10% or no fifth instars appearing after 12 days on 1 or 5 µg/g OTA-supplemented diets and 40% fifth instars appearing on the 0.25% DMSO control diet (p = 0.032 or 0.015, respectively) (Fig. 2b).

3.3 Metabolism of AFB1 and OTA by NOW

To investigate the ability of A. transitella to metabolize AFB1, in vitro metabolism experiments were conducted with cleared midgut lysates quantified based on total protein. In reactions initiated with NADPH (as outlined in Materials and Methods), two polar metabolites of AFB1 (Met1 with retention time of 7.8 min and Met2 with retention time of 8.0 min) were readily detected by HPLC analysis (Data not shown). Compared with H. zea midgut lysates, A. transitella midgut lysates have much higher turnover rates (32 pmol/min/mg total protein vs. 0 pmol/min/mg total protein). In contrast, CEW midguts show no activity toward AFB1 unless they were induced by prior exposure to coumarin and xanthotoxin (Niu et al., 2008).

In an attempt to trap reactive AFBO intermediates, mouse liver cytosol was added to some in vitro reactions but no additional metabolite peaks were detected (data not shown). This finding is consistent with the suggestion that AFB1 is detoxified, and not bioactivated, as the result of hydroxylation by midgut proteins. Comparable reactions conducted with OTA showed no metabolism of OTA by A. transitella and H. zea lysates either at the level of substrate disappearance or metabolite production. The methods used in this analysis may have been insufficiently sensitive to detect metabolism; alternatively, both of these species may rely on detoxification in tissues other than midgut, or non-enzymatic means, to counter the toxic effects of this compound.

4. Discussion

Zeng et al. (2006) and Niu et al. (2008) studied the toxicity and metabolism of AFB1 by H. zea, a lepidopteran with tolerance to a broad range of phytochemicals but only limited tolerance to aflatoxins even though it occasionally encounters aflatoxin-releasing fungi. In contrast, A. transitella, a herbivore that specifically
locates and feeds on mummy fruits, is tolerant of extremely high concentrations of mycotoxins in its diet. Our studies indicate that *A. transitella*, although a hostplant generalist, may well be highly specialized for mycotoxin detoxification. Indeed, recent studies indicate that NOW grows better on fungus-contaminated hostplant tissue and may in fact consume fungus tissue as well (Palumbo et al., 2008; personal observations).

In this study, we demonstrated the extremely high tolerance of *A. transitella* to concentrations of AFB1 and OTA and showed that this tolerance exceeds that of other mycotoxin-associated species such as *H. zea*. The concentrations analyzed did not kill *A. transitella* when administered at early developmental stages and caused only marginal sublethal effects. Comparisons of the toxicological effects in *A. transitella* and *H. zea* demonstrated that *A. transitella* larvae are 100 times more tolerant of AFB1 than *H. zea* and are also significantly more resistant to OTA. The ecological consequences of this high tolerance are quite apparent in the high survival rates of *A. transitella* larvae in the mycotoxin-contaminated environments that it exploits as a scavenger. The interaction between NOW and *Aspergillus* species might be mutualistic in that the fungus may contribute to maintaining a micro-environment that optimizes caterpillar growth and development and the caterpillar may promote the dispersal and establishment of the fungus by causing damage that allows fungal spores to gain access to fruits (Weis, 1982). *Aspergillus* species can be cultured from adults and the levels are high in adults emerging from *Aspergillus*-infested substrate (JS, unpublished data).

High tolerance of aflatoxins might be explained by an excess detoxification capacity that generates nontoxic metabolites. Both Lee and Campbell (2000) and this study demonstrated that no bioactivated metabolites are produced in the reactions of NOW proteins. Because AFBO production increases toxicity 100-fold compared to its parent compound (AFB1) (Eaton and Gallagher, 1994; Guengerich et al., 1998; McLean and Dutton, 1995; Suriawinata and Xu, 2004), absence of AFBO production by NOW might be a key feature of AFB1 tolerance. In contrast with AFB1, there are reports of detoxification of OTA in insects. Although
we failed to detect metabolites of OTA by midgut proteins of either *H. zea* or *A. transitella*, bioactivation is involved in its genotoxicity to other organisms (Manderville, 2005; Schaut et al., 2008) and the absence of major pathways producing bioactivated metabolites may also reflect adaptation of these insects to the presence of this mycotoxin in their diet.
References


Weis AE, 1982. Use of a symbiotic fungus by the gall maker *Asteromyia carbonifera* to inhibit attack by the parasitoid, *Torymus capite*. Ecology, 63:1602-1604


Widstrom NW, 1979. The role of insects and other plant pests in aflatoxin contamination of corn, cotton, and peanuts--a review. *J. Environ. Qual.*, 31

Table 1. Pupation rate and pupal weight of *A. transitella* larvae after exposure to different concentrations of aflatoxin B1 (in 0.5% DMSO) at first instar. Values for pupation rate are means ± standard errors from 20 first instar caterpillars/treatment with three experimental replicates for each. Values for pupal weight are means of three series of replicates with standard errors.

<table>
<thead>
<tr>
<th>Concentration (µg/g)</th>
<th>Pupation rate (%)</th>
<th>Pupal weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% DMSO</td>
<td>85 ± 6</td>
<td>43 ± 2</td>
</tr>
<tr>
<td>1</td>
<td>90 ± 5</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>5</td>
<td>85 ± 6</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>10</td>
<td>77 ± 9</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>20</td>
<td>78 ± 2</td>
<td>44 ± 3</td>
</tr>
<tr>
<td>50</td>
<td>55 ± 10 *</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* indicates that the mean value of this group is significant different from that of the control group (0.5% DMSO) (p < 0.05).
Table 2. Toxicity of aflatoxin B1 to ultimate (fifth) instar *A. transitella*

Values for pupation rate are means ± standard errors from 20 ultimate instars/treatment with three experimental replicates for each. Values for larval and pupal weight are means of the three series of replicates with standard errors. The data were evaluated with one-way analysis of variance (ANOVA) and no differences were found in the means of pupation rate or pupal weight among the groups.

<table>
<thead>
<tr>
<th>Concentration (µg/g)</th>
<th>Pupation rate (%)</th>
<th>Pupal weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>plain diet</td>
<td>97 ±6</td>
<td>41 ±4</td>
</tr>
<tr>
<td>0.5% DMSO</td>
<td>98 ±3</td>
<td>38 ±3</td>
</tr>
<tr>
<td>50</td>
<td>92 ±10</td>
<td>38 ±4</td>
</tr>
<tr>
<td>100</td>
<td>93 ±3</td>
<td>39 ±4</td>
</tr>
</tbody>
</table>
Table 3. Toxicity of ochratoxin A to first instar larvae of *A. transitella*
Values for survival rate are means ± standard errors from three replicate bioassays. Values for larval and pupal weight are means of the three series of replicates with standard errors.

<table>
<thead>
<tr>
<th>Concentration (µg/g)</th>
<th>Survival rate on day 12 (%)</th>
<th>Larval weight on day 14 (mg)</th>
<th>Pupation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain diet</td>
<td>90 ± 10</td>
<td>38 ± 2</td>
<td>90 ± 7</td>
</tr>
<tr>
<td>0.25% DMSO</td>
<td>85 ± 5</td>
<td>36 ± 2</td>
<td>75 ± 4</td>
</tr>
<tr>
<td>1</td>
<td>80 ± 10</td>
<td>32 ± 9</td>
<td>80 ± 7</td>
</tr>
<tr>
<td>5</td>
<td>85 ± 5</td>
<td>33 ± 3</td>
<td>83 ± 5</td>
</tr>
<tr>
<td>10</td>
<td>85 ± 10</td>
<td>35 ± 5</td>
<td>75 ± 4</td>
</tr>
<tr>
<td>20</td>
<td>90 ± 5</td>
<td>33 ± 2</td>
<td>78 ± 2</td>
</tr>
<tr>
<td>50</td>
<td>80 ± 5</td>
<td>26 ± 6*</td>
<td>75 ± 4</td>
</tr>
</tbody>
</table>

* indicates that the mean value of this group is significant different from that of the control group (0.5% DMSO) (p < 0.05).
Fig. 1 Developmental effects of AFB1 on first instar *H. zea* and *A. transitella.* (A) Percentage of *H. zea* larvae molting to second instar after 48 hr on diets containing increasing concentrations of AFB1 and a final concentration of 0.2% DMSO. (B) Percentage of *A. transitella* larvae molting to second instar after 48 hr on diets containing increasing concentrations of AFB1 and a final concentration of 0.2% DMSO.
Fig. 2 Toxicity of OTA to first instar larvae of *H. zea*. (A) Survivorship of larvae 12 days post-hatch on diets containing increasing concentrations of OTA and a final concentration of 0.25% DMSO. (B) Frequency of fifth instar larvae 12 days post-hatch on diets containing 0.25% DMSO, 1 µg/g OTA in 0.1% DMSO or 5 µg/g OTA in 0.25% DMSO.
III Toxicity of mycotoxins to honey bees and its amelioration by propolis

Abstract

Honey bees and their resource-rich nests are hosts to a wide range of saprophytic fungi, including species that produce mycotoxins. The toxicity of aflatoxin B1 (AB1) and ochratoxin A (OTA), products of Aspergillus species often found in honey bee hives, was evaluated and LC50 values for both toxins were calculated. Workers can tolerate a wide range of concentrations of both OTA and AB1. At low concentrations, AB1 (1μg/g and 2.5μg/g diet) and OTA (1μg/g ) did not have any apparent toxic effects on bees. Enhancement of the toxicity of AB1 by piperonyl butoxide, a known inhibitor of cytochrome P450 monooxygenases (P450s), indicates a role for P450s in AB1 detoxification in honey bees. Extracts of propolis, a complex mixture of plant-derived chemicals, including many flavonoids and other phenolic compounds, similarly ameliorated aflatoxin toxicity and delayed the onset of mortality. Collectively, these results suggest that tolerance of AB1 by honey bees may be due to P450-mediated metabolic detoxification.
1. Introduction

Although honey bees are more sensitive to the toxic effects of the mycotoxin aflatoxin B1 than *Drosophila melanogaster* and *Musca domestica* (Hildrup and Llewellyn, 1978), the fact that colonies are often able to fend off infections is indicative of some capacity for coping with these mycotoxins, which may be frequently encountered in the hive environment. In many insect species, aflatoxins are metabolized by cytochrome P450 monooxygenases (P450s), heme-based enzymes that generally catalyze oxidative reactions that reduce lipophilicity and hence toxicity of a wide range of both natural and synthetic xenobiotics (Li et al., 2007). Aflatoxins and several other mycotoxins are unusual, however, in that in many species P450-mediated metabolic reactions result in bioactivation, or an increase in toxicity, via conversion of the aflatoxin to the more toxic epoxide metabolite (Saner et al., 1996).

Species that naturally encounter aflatoxins in their environment display some degree of adaptation to mycotoxins in that they metabolize these compounds not to bioactivated epoxides but rather to nontoxic breakdown products. The corn earworm *Helicoverpa zea*, for example, frequently causes damage in its hostplants that leaves them vulnerable to opportunistic infection by *Aspergillus* spp. In the midgut, *H. zea* detoxifies aflatoxin B1 (AB1) via CYP321A1 (Niu et al., 2008). Even more tolerant of aflatoxins is the navel orangeworm *Amyelois transitella*. This species infests dried fruits and nuts, particularly almonds and pistachios, and appears to prefer fungus-infected fruit (Palumbo et al., 2008). This species can tolerate dietary levels of AB1 100-fold greater than levels that inhibit *H. zea* (Niu et al., 2009) and produces principally aflatoxicol as a metabolite. Neither this species nor the aflatoxin-tolerant codling moth *Cydia pomonella* produces AB1-8,9-epoxide, the principal bioactivated metabolite of AB1 (Lee and Campbell, 2000). Although P450-mediated metabolism has been implicated in this tolerance, the specific P450(s) responsible for this detoxification have not yet been identified in either species.

In this study, we set out to ascertain the degree to which *A. mellifera* can tolerate exposure to mycotoxins and to establish whether tolerance is associated with
P450-mediated metabolism by using a known inhibitor of honey bee P450s, piperonyl butoxide (PBO), and a known inducer of honey bee P450s, propolis, the resinous “bee glue” that is a ubiquitous component of the hive; Johnson (2008) demonstrated that extracts of propolis administered orally to honey bees effects upregulation of three CYP6AS P450 genes, at least one of which is known to metabolize flavonoids found in honey, pollen and propolis (Mao et al. 2009). Understanding the mechanisms of resistance to mycotoxins may shed light on how this managed pollinator copes with chemical stresses.

2. Materials and Methods

2.1 Insects

Honey bees were obtained from colonies containing multiply mated queens at the University of Illinois Bee Research Facility at Urbana (Champaign County), IL. Frames of late-stage pupae were taken from hives and transferred to a dark humid incubator at 32-34°C. Newly eclosed adults were brushed from frames every 24 hours for immediate use in bioassays.

2.2 Chemicals

Aflatoxin B1 (AB1) and ochratoxin A (OTA) were obtained from Sigma Co (St Louis, MO). PBO was purchased from Tokyo Kassie Kogyo (Tokyo, Japan). Analytical grade dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA). All solvents, including DMSO and methanol, were of analytical reagent grade. AB1 and OTA were dissolved in DMSO as concentrated stock solutions and stored in a –20°C freezer.

2.3 Substrate carrier

Chemicals to be tested were administered to bees by incorporation into “bee candy,” made using equal weights of powdered sugar and concentrated sugar solution with a ratio of 2:1 sucrose to water (w/w). Granulated table sugar (sucrose) was ground in a blender for approximately 3 min to make powdered sugar. Approximately 5 g fresh candy was poured into a 2 oz (56 ml) plastic cup (Solo, Urbana, IL); mycotoxins or a vehicle control were incorporated into the wet candy, which was then allowed to harden for 30 min.
To test effects of propolis on aflatoxin toxicity to honey bees, propolis was collected from hives in a forested area at Phillips Tract Research Area (Champaign Co., Illinois). Propolis was frozen in liquid nitrogen and then ground with a mortar and pestle and stored at -20º C. Approximately 3 g of ground propolis was dissolved in 50 ml of hot methanol. Wax was precipitated from the cooled methanol extract and the remaining extract was concentrated to 5 ml by evaporation under a stream of air. Propolis solution was added to powdered sugar and the methanol was allowed to dry for 24 h before mixing with an equal mass of heavy sugar syrup to make candy with a final concentration of 50, 150 and 300 mg propolis/g candy.

2.4 Bioassays

Newly eclosed workers were used for bioassays. Stocks of 10 µg/g or 20 µg/g of AB1 and OTA were dissolved in DMSO and stored at -20º C. Bee candy was used to test the oral toxicity of mycotoxins with and without synergists via bioassay. For all bioassays, approximately 3 g of unanaesthetized newly emerged bees were transferred quickly to a 6 oz (188 ml; Sweetheart, Owings Mills, MD) wax-coated paper cup into which the treated candy had been placed. The cups were then covered with cheesecloth and placed into a dark humid incubator at a temperature of 32-34º C. The mortality of bees was recorded every six hours until all bees had died.

To determine the oral LC50 of AB1 to bees, AB1 was incorporated into candy to achieve levels of 0.5, 1, 2.5, 5, 7, 10, 15 or 20 µg AB1 per g candy; in an additional treatment, DMSO was added to bee candy as a control. To determine the LC50 of OTA to bees, candy containing 1, 5, 10, 20, 40, 60 or 80 µg/g OTA was prepared, along with control candy containing DMSO. To test the effects of the known P450 synergist, (PBO, on the toxicity of AB1 or OTA to bees, two concentrations of PBO (0.05% or 0.1% PBO) were tested in the presence of AB1 or OTA. For AB1 vs PBO bioassays, candy containing six different concentrations of chemicals was prepared: 0.1% DMSO, 10 µg/g AB1, 0.05% PBO, 0.1% PBO, 10 µg/g AB1 supplemented with 0.05% or 0.1% PBO. For the bioassay comparing OTA and PBO, two concentrations of OTA (10 and 40 µg/g) were tested and compared
with corresponding concentrations of OTA supplemented with 0.05% or 0.1% PBO.

To determine the effects of propolis, which has been shown to induce a subset of P450 enzymes involved in detoxification (Johnson, 2008), the toxicity of AB1 and OTA in propolis-treated candy was determined. Five µl of stock AB1 (20µg/µl) was incorporated into 5g of plain candy to prepare the candy containing 20 µg/g AB1 or into 5 g of candy containing 50, 150 and 300 mg/g of propolis and mixed until the toxin was homogeneously distributed in the fresh candy. Candy treated with DMSO only and candy containing 50, 150 or 300 mg/g propolis without AB1 were used as controls.

2.5 Statistics

The R statistical package (R Development Core Team, 2009) with MASS libraries (Venables and Ripley, 2002) was used for log-probit analysis. LC$_{50}$ values with 95% confidence intervals were calculated using Fieller’s method (Finney, 1971). Treatments with non-overlapping 95% confidence intervals for LC$_{50}$ were considered significantly different. Survival data to determine effects of PBO or propolis on the toxicity of AB1 or OTA to bees were analyzed using SPSS statistical software (SPSS Inc., Chicago, IL). With the function of survival analysis in SPSS, the median time for survival was calculated for each treatment independently. To compare the data among groups in any given bioassay, the data from all treatments were compared with one-way analysis of variance (Tukey plot) using the function of survival analysis in the software package. In the bioassay testing the effects of PBO on the toxicity of AB1 or OTA to honey bees, the p value was calculated to compare effects of AB1 or OTA in the presence or absence of the inhibitor. In the bioassay testing the effects of propolis extracts on the toxicity of AB1, the p value was calculated to compare impacts of AB1 in the presence and absence of propolis. Comparisons yielding p values equal to or less than 0.05 were considered to indicate significant differences.

3. Results

3.1 Toxicity of mycotoxins to A. mellifera

None of the treatments containing AB1, even at the highest dose of 20
μg/g, resulted in bee mortality in less than 24 hours (Fig. 1). The low concentrations of AB1 (0.5, 1μg and 2.5μg/g) did not have any apparent toxic effects on bees over the entire duration of the bioassays. The intermediate and high doses of AB1 (5μg/g and 7μg/g) caused less than 50% mortality after 72 hours. The high doses of AB1 (10, 15 and 20μg/g) caused over 90% mortality in 72 hours (Fig. 1). The LC₅₀ of AB1 (defined as the concentration causing 50% mortality of treated bees in 72 hours) was 6.76 μg/g (95% conf. int.= 5.86-7.69; slope=4.32+/−0.30; X²=6.67; df=4; n=590) (Fig. 3).

Bees also displayed tolerance of a wide range of concentrations of OTA. The low concentration of OTA (1μg/g) did not have any apparent toxicity to bees and the treated bees lived as long as did the control bees. As with the AB1 treatments, no bee mortality was observed, even at the highest concentrations, in less than 24 hours. Over time, mortality increased and concentrations equal to or greater than 10μg/g caused 100% mortality after 72 hours. Most of treated bees died during the final 24 hours (from 48 hours to 72 hours) (Fig. 2). The LC₅₀ of OTA at 72 hours for bees was 5.04μg/g (95% conf. int.=1.73-7.90; slope=1.11+/−0.28; X²=1.49; df=2; n=210) (Fig. 3).

While both OTA and AB1 exhibit similar toxicity to bees, the slope of the probit lines for AB1 is substantially steeper than is the slope of the probit line for OTA, indicating that the response to OTA is more heterogeneous than the response to AB1.

3. 2 Effects of synergists on the toxicity of AB1 or OTA to bees

Enhancement of the toxicity of AB1 and OTA by administration of PBO, a known inhibitor of P450s in bees (Johnson et al., 2008), indicates a role for P450s in mycotoxin metabolism in honey bees. Bees started to die earlier when consuming candy containing 10μg/g AB1 supplemented either with 0.05% PBO or 0.1% PBO than did bees consuming candy supplemented with 10μg/g AB1 alone (Table 1). These results suggest that P450s contribute to enzymatic detoxification of AB1 in honey bees.

In contrast with AB1, PBO did not show any synergistic effects on the
toxicity of OTA to bees. Two concentrations of PBO (0.05% and 0.1% PBO) were applied and two concentrations of OTA (10 and 40μg/g) were used in the bioassays. There was no indication that PBO can accelerate or delay death of bees fed OTA (Table 1).

3.3 Effects of propolis on the toxicity of AB1

To determine whether propolis, as an inducer of P450s, may contribute to tolerance of mycotoxins, bioassays of bees fed with AB1 alone or AB1 and propolis were conducted. Bees consuming candy containing 20μg/g AB1 started to die after 24 hours and all had died after 60 hours, whereas bees consuming candy containing the same concentration of AB1 supplemented with any amount of propolis lived longer than bees fed with AB1 alone; fewer than 10% of bees died in 60 hours (data not shown). Survival analysis shows that the median survival time of bees fed with 20μg/g AB1 is 44 hours while that of bees fed with candy containing 20 μg/g AB1 plus 50, 150 or 300 mg/g propolis is 126, 150 and 154 hours, respectively. There is a significant difference in survival time between bees treated with AB1 and those treated with AB1 and propolis (Table 2). Longer median survival time with higher concentrations of propolis (150 mg/g and 300 mg/g) suggests a dosage-dependent ameliorative effect of propolis on AB1 toxicity.

4. Discussion

As repositories of large quantities of food resources maintained under conditions of high relative humidities and temperatures, beehives are particularly vulnerable to opportunistic fungal infections. Given the frequency with which fungi such as Aspergillus colonize such environments, the relative tolerance that A. mellifera displays to mycotoxins such as AB1 and OTA is ecologically consistent with the likelihood of exposure. Similarly, H. zea, the corn earworm, which only intermittently encounters aflatoxins in its preferred foodplants, displays substantially greater sensitivity to AB1 than does A. transitella, the navel orangeworm, which routinely (and possibly preferentially) consumes fungus-contaminated plant food.

How A. mellifera tolerates mycotoxins such as aflatoxins and ochratoxins has not yet been determined definitively. In aflatoxin-tolerant species such as H. zea
and *A. transinitella*, metabolism of AB1 to non-toxic products is mediated by P450s (Niu et al., 2008); in species that are less tolerant, such as *Drosophila melanogaster*, P450-mediated metabolism appears to result in epoxidation and hence bioactivation, or enhanced toxicity (Saner et al., 1995). That administration of PBO in the presence of aflatoxin increased toxicity suggests that the honey bee, like the aflatoxin-tolerant *H. zea* and *A. transinitella*, transforms these compounds to nontoxic metabolites via P450-mediated metabolism. Recent work by Johnson et al. (2009) indicates that the organophosphate coumaphos is also metabolized via P450s in bees to nontoxic products, rather than bioactivated to the more toxic oxon metabolite, as is the case for most insects.

Ochratoxins are also produced by fungi that can be found in hive products (Gilliam et al., 1989). We found that, although workers can tolerate low levels of OTA (1 µg/g), concentrations higher than 5 µg/g will kill over 50% of exposed bees in 3 days under bioassay conditions. To date, no studies have been published on OTA metabolism by insects. Although metabolism of OTA in mammals is mediated primarily by P450s (Neal, 1995), the fact that, in our study, PBO has no effects on OTA toxicity suggests that this enzyme system might not be involved in the detoxification of OTA in honey bees. Studies with synergists must be interpreted cautiously, however, inasmuch as PBO does not necessarily inhibit all P450-mediated transformations in insects (Sanchez-Arroyo et al., 2001).

Propolis, prepared from resinous materials collected from plants by honey bees, is thought to function principally as a sealant for gaps within the hive to enhance structural stability (Burdock, 1998; Bankova, 2005). It also plays a key role in reducing decay of extraneous organic material within the hive and may also help bees fend off parasites and pathogens. Its antimicrobial activity is attributed to its high content of flavonoids and phenolics and in fact propolis extracts *in vitro* can inhibit the growth of fungi, including *Aspergillus versicolor*, *A. flavus*, *A. sulphureus* and *A. parasiticus*, and suppress production of mycotoxins, including AB1, OTA and sterigmatocystin (Aly and Elewa, 2007; Gómez-Caravaca et al., 2006; Ozcan 2004; Pepeljnjak et al. 1982; Viuda-Martos et al., 2008). Simone et al. (2009) recently
showed that exposure to propolis extracts downregulates expression of two honey bee immune-function genes concomitant with lowering the eubacterial loads, suggesting that propolis in the hive may reduce the need for immune gene expression by reducing bacterial loads.

That propolis may contribute to honey bee defense against fungi and their associated toxins not only by suppressing microbe growth but also by enhancing detoxification enzymes is a novel hypothesis consistent with the results of our study, at least with respect to AB1. Of the 46 P450s that are found in the honey bee genome, several in the CYP6AS subfamily are selectively induced by hive products such as honey, pollen and propolis, indicating a possible role in xenobiotic detoxification (Johnson, 2008); indeed, Mao et al. (2009) demonstrated that CYP6AS3 is capable of metabolically transforming quercetin, a flavonoid found in propolis and in a wide variety of honeys. The ability of propolis to ameliorate the toxicity of AB1, as demonstrated in this study, suggests a function for propolis as an adjuvant for detoxification. Although the honey bee genome contains far fewer P450 genes than do other sequenced insect genomes, bees may compensate for reduced numbers by efficiently regulating the expression of a small number of P450s for detoxification by induction with dietary phytochemicals. Individual P450s might be greatly induced by propolis extracts to bio-transform AB1 into less toxic compounds, thereby reducing the risks of exposure to mycotoxins.

Propolis has long been used in traditional medicine and has been credited with immuno-modulatory, anti-inflammatory and antimicrobial activity. At least some of the therapeutic activity of propolis in alternative medicine has been attributed to its function as an inducer of P450 activity (Bhaudauria et al., 2007). Propolis treatment has been shown to increase activities of a range of P450s, including pentoxyresorufin depentylase, and ethoxycoumarin deethylase (Siess et al. 1996). More recently, Beltrán-Ramírez et al. (2008) reported that caffeic acid phenethyl ester, a common constituent of propolis, can modify expression of multiple P450s to inhibit the activation of diethylnitrosamine in rats. To some extent, it is remarkable that the body of knowledge relating to the biochemical activity of propolis in human health is
substantially larger than that relating to the biochemical activity of propolis in honey bee health; a greater understanding of the multiple roles of propolis in the life of the honey bee can be gained by further study of the ways in which it is processed and metabolized.

References


Table 1. Survival analysis of effects of piperonyl butoxide (PBO) on toxicity of AB1 or OTA to bees. Bioassays were conducted to compare survival time of groups of bees fed mycotoxins in the presence and absence of PBO. Estimated median for survival time of bees fed “candy” containing 10 µg/g AB1, 10 µg/g or 40 µg/g OTA, 0.05% PBO, 0.1% PBO, 10 µg/g AB1 supplemented with 0.05% PBO or 0.1% PBO, 10 µg/g or 40 µg/g OTA supplemented with 0.05% PBO or 0.1% PBO is the average of three replicates. Treatments of PBO plus toxin that differ significantly from toxin alone are indicated with “*” (p < 0.05).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Median for survival time (hours)</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% DMSO</td>
<td>186</td>
<td>25</td>
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<tr>
<td>0.05% PBO</td>
<td>154</td>
<td>19</td>
</tr>
<tr>
<td>0.1% PBO</td>
<td>168</td>
<td>6</td>
</tr>
<tr>
<td>10 µg/g AB1</td>
<td>66</td>
<td>17</td>
</tr>
<tr>
<td>0.05% PBO plus 10 µg/g AB1*</td>
<td>39</td>
<td>4</td>
</tr>
<tr>
<td>0.1% PBO plus 10 µg/g AB1*</td>
<td>39</td>
<td>4</td>
</tr>
<tr>
<td>10 µg/g OTA</td>
<td>75</td>
<td>4</td>
</tr>
<tr>
<td>0.05% PBO plus 10 µg/g OTA</td>
<td>72</td>
<td>0</td>
</tr>
<tr>
<td>0.1% PBO plus 10 µg/g OTA</td>
<td>72</td>
<td>8</td>
</tr>
<tr>
<td>40 µg/g OTA</td>
<td>57</td>
<td>4</td>
</tr>
<tr>
<td>0.05% PBO + 40 µg/g OTA</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>0.1% PBO + 40 µg/g OTA</td>
<td>60</td>
<td>0</td>
</tr>
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Table 2. Survival analysis of effects of propolis on toxicity of aflatoxin B1 (AB1) to bees. Bioassays were conducted to compare survival time of groups of bees fed mycotoxins in the presence of different concentrations of propolis. Estimated median survival time of bees fed “candy” containing 20 µg/g AB1, 50 mg/g, 150 mg/g and 300 mg/g propolis, and 20 µg/g AB1 supplemented with either of 50 mg/g, 150 mg/g or 300 mg/g propolis is the average of three replicates. Survival times for groups feeding on propolis and AB1 that are significantly different from survival time of groups consuming AB1 alone are indicated with “*”.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Median for survival time (hours)</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% DMSO</td>
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</tr>
<tr>
<td>50 mg/g propolis</td>
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<td>150 mg/g propolis</td>
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<td>300 mg/g propolis</td>
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<td>20 µg/g AB1</td>
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<td>50 mg propolis plus AB1</td>
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<td>8</td>
</tr>
<tr>
<td>300 mg propolis plus AB1</td>
<td>154</td>
<td>18</td>
</tr>
</tbody>
</table>
Fig. 1 Mortality of *Apis mellifera* exposed to different concentrations of dietary aflatoxin B1 (AB1). Groups of approximately 30 newly enclosed workers were tested with “bee candy” containing a range of concentrations of AB1 in DMSO and candy containing DMSO only. Each experiment was repeated three times; this figure graphically depicts the result of one replicate for visual clarity.
Fig. 2 Mortality of *Apis mellifera* exposed to different concentrations of dietary ochratoxin A (OTA). Groups of approximately 30 newly enclosed workers were exposed to “bee candy” containing a range of concentrations of OTA in DMSO and control containing DMSO only. Each experiment was repeated three times; this figure graphically depicts the result of one replicate for visual clarity.
Fig. 3 Comparison of LC$_{50}$ of AB1 and OTA to bees. Plots of probits of bee mortality relative to different concentrations of AB1 or OTA. A (red) depicts AB1 treatments; O (black) depicts OTA treatments. LC$_{50}$ is defined as the concentration of toxin that can cause 50% mortality of the tested bees after 72 hours. Each experiment was repeated three times and the estimated LC$_{50}$ is the average of three replicates. The calculated LC$_{50}$ for AB1 and OTA are 6.79 µg/g and 5.04 µg/g.
IV Aflatoxin B1 detoxification by CYP321A1 in *Helicoverpa zea*

Abstract

The polyphagous corn earworm *Helicoverpa zea* frequently encounters aflatoxins, mycotoxins produced by the pathogens *Aspergillus flavus* and *A. parasiticus*, which infect many of this herbivore’s hostplants. While aflatoxin B1 metabolism by midgut enzymes isolated from fifth instars feeding on control diets was not detected, this compound was metabolized by midgut enzymes isolated from larvae consuming diets supplemented with xanthotoxin, coumarin, or indole-3-carbinol, phytochemicals that are likely to co-occur with aflatoxin in infected hostplants. Of the two metabolites generated, the main derivative identified in midguts induced with these chemicals and in reactions containing heterologously expressed CYP321A1 was aflatoxin P1 (AFP1), an O-demethylated product of AFB1. RT-PCR gel blots indicated that the magnitude of CYP321A1 transcript induction by these chemicals is associated with the magnitude of increase in the metabolic activities of induced midgut enzymes (coumarin>xanthotoxin>indole 3-carbinol). These results indicate that induction of P450s, such as CYP321A1, plays an important role in reducing AFB1 toxicity to *H. zea*. Docking of AFB1 in the molecular models of CYP321A1 and CYP6B8 highlights differences in their proximal catalytic site volumes that allow only CYP321A1 to generate the AFP1 metabolite.
1. Introduction

Cytochrome P450 monooxygenases (P450s) comprise a large superfamily of hemoproteins that contribute to detoxification in virtually all aerobic organisms, including insects (Feyereisen, 2005). Within every genome, the multiplicity of P450s with potentially broad substrate specificity presents a challenge with respect to identifying ecologically relevant substrates. While some P450-mediated reactions convert chemical(s) to more toxic substances, many more mediate the detoxification of toxic chemical(s) into less toxic metabolites. Depending on the proportions of each type of P450 expressed in a particular tissue or developmental stage, compounds can either be bioactivated or detoxified (McLean and Dutton, 1995; Neal, 1995; Guengerich, 2006). In some cases, as in the vertebrate P450-mediated metabolism of aflatoxin B1, a fungal toxin produced by Aspergillus flavus and Aspergillus parasiticus, a single compound can be both detoxified and bioactivated by a single P450 (Gallagher et al., 1996; Johnson et al., 1997; Guengerich et al., 1998).

Characterizing ecologically significant P450 substrates is also complicated by the fact that expression of individual P450 genes can be up- or down-regulated by different compounds ingested by the organism. For herbivorous insects, regulation of P450 expression by phytochemicals and other dietary constituents is of particular importance in that herbivores encounter these compounds in a variety of combinations, depending on the nature of the plant tissue ingested (Zeng et al., 2007). The ecological consequences of such phytochemical regulation depend on the P450s involved and the toxins encountered by herbivores subsequent to phytochemical exposure: up-regulation of detoxicative processes is likely to be beneficial to the herbivore whereas down-regulation of detoxification and up-regulation of bioactivation are likely to be detrimental to the herbivore.

Among the dietary constituents whose metabolism may be affected by co-occurring phytochemicals are mycotoxins produced by plant pathogens. Contamination of crops by aflatoxins causes losses of millions of dollars annually in the United States primarily because their toxic effects make contaminated grains unsuitable for livestock and human consumption (Robens and Cardwell, 2005).
Studies in vertebrate systems have indicated that the toxicity of AFB1 is derived from its conversion by P450s and lipoxygenases to AFB1-8,9-\textit{exo}-epoxide (AFBO), a highly reactive compound that interacts with DNA to form guanyl N7-adducts capable of inhibiting DNA synthesis and generating point mutants that activate protooncogenes, inactivate cancer suppressor genes, and/or alter protein functions (Eaton and Gallagher, 1994; McLean and Dutton, 1995; Guengerich et al., 1998). In these systems, detoxification of AFB1 is achieved by several P450-mediated routes, including the formation of hydroxylated metabolites such as AFM1, AFQ1, AFB2a and AFP1, which have lower or no ability to form the 8,9-\textit{exo}-epoxide (AFBO).

AFB1 can also be directly reduced to aflatoxicol (AFL) by NADPH-dependent reductases but this derivative is rapidly converted back to AFB1 by a dehydrogenase (Eaton and Gallagher, 1994). The highly mutagenic AFBO formed in some vertebrate systems (but not any known insect systems) can be detoxified by glutathione S-transferase (GST)-mediated conjugation with glutathione (GSH) and, often, the sensitivity of different mammalian species to AFB1 is associated with the activities of their GSTs (Wong and Hsieh, 1980; Degen and Neumann, 1981; Lotlikar et al., 1984).

\textit{Aspergillus} infections of crops, with concomitant aflatoxin contamination, are often exacerbated by infestations of insects such as \textit{Helicoverpa zea} (Dowd and White, 2002), a broadly polyphagous species associated with a wide range of crop species (Neunzig et al., 1963; Archer and Bynum, 1994; Capinera, 2001). In contrast with vertebrates, little is known about AFB1 biotransformations in insects. While no direct \textit{in vitro} or \textit{in vivo} evidence exists for the transformation of AFB1 to AFBO in insects, several studies have indirectly suggested that insect P450s bioactivate AFB1. In particular, Saner et al. (1996) demonstrated that \textit{Drosophila melanogaster} CYP6A2 is capable of transforming AFB1 into a product that induces gene conversion. Zeng et al. (2006) demonstrated that addition of a P450 inhibitor, piperonyl butoxide (PBO), to \textit{H. zea} diets containing 1 µg/g AFB1 significantly decreases the toxicity of AFB1 to fourth instars and increases the pupation rate compared to that of larvae on diets containing only AFB1. In contrast with this
apparent bioactivation of AFB1, several detoxified metabolites, including AFM1, AFB2a and AFL, have been identified in *in vitro* metabolism experiments conducted with *D. melanogaster*, *Amyelois transitella* (navel orangeworm) and *Cydia pomonella* (codling moth) (Foerster and Wurgler, 1984; Lee and Campbell, 2000).

Li et al. (2002a,b) and Zeng et al. (2007) have shown that defenses against naturally occurring and synthetic xenobiotics can be dramatically enhanced in *H. zea* following ingestion of certain phytochemicals. Relevant to this study, a variety of plant phytochemicals, including xanthotoxin, coumarin, and indole-3-carbinol, significantly reduced the toxicity of AFB1 to *H. zea*, as evidenced by increased pupation rates and pupal weights. Molecular analyses have indicated that several members of the *H. zea* CYP6B subfamily (CYP6B8, CYP6B27, CYP6B28) and CYP321A1 are differentially regulated by these compounds (Li et al., 2002a,b, 2004; Sasabe et al., 2004; Zeng et al., 2007). With CYP6B8 and CYP321A1 known to metabolize an array of phytochemicals and insecticides (Li et al., 2002a,b, 2004; Sasabe et al., 2004; Rupasinghe et al., 2007), we examined the mode of AFB1 detoxification induced by phytochemicals in this species. Our results indicate that at least one P450 up-regulated 8- to 20-fold by these chemicals, CYP321A1, metabolizes AFB1 into two products, with the major product corresponding to the less toxic AFP1.

2. Materials and Methods

2.1 Chemicals and biological materials

The aflatoxin standards (AFB1, AFB2, AFM1, AFG1, AFG2), plant allelochemicals (indole-3-carbinol, coumarin, xanthotoxin) were purchased from Sigma Chemical Co. (St. Louis, MO). Analytical grade dimethyl sulfoxide (DMSO) and piperonyl butoxide (PBO) were purchased from Fisher Scientific (Hampton, NH). All of these compounds were dissolved in DMSO as stocks stored in –20°C. A laboratory strain of *H. zea* provided by Abbott Laboratories (Chicago, IL) was maintained in an insectary kept at 23-26°C with a photoperiod of 16 hr light: 8 hr dark. *H. zea* larvae were individually reared in 30 ml creamer containers on a semisynthetic diet containing wheat germ (Waldbauer et al., 1984). Mouse livers
were provided by Renhao Lai and Dr. Elizabeth Jeffery (UIUC) and purified rat liver glutathione-S-transferase proteins were obtained from Sigma Chemical (St. Louis, MO). Mice were anesthetized by using CO₂, followed by cervical dislocation. Livers were collected immediately and frozen on dry ice and stored at -80°C until use. All mouse procedures were approved by the UIUC Animal Care and Use Committee and were conducted in accordance with its policies.

2.2 Preparation of insect and mouse microsomal and cytosolic fractions

A group of 20 fifth instars that had all molted within the same 12 hr period were fed with artificial diets containing 0.2% DMSO (control), 1 mg/g indole-3-carbinol, 1 mg/g coumarin, 0.5 mg/g xanthotoxin, 1 µg/g AFB1 for 48 hr. Midguts were then dissected in ice-cold 0.1 M phosphate buffer (pH 7.8) and stored at -80 °C. The frozen midguts were ground to a fine powder using mortars and pestles in the presence of liquid N₂. Each powdered midgut sample derived from 20 larvae was then transferred into a 2 ml glass homogenizer containing 1.5 ml resuspension buffer (0.1 M phosphate buffer (pH 7.8), 1 mM EDTA, 0.5 mM PMSF, 0.1 mM DTT, 20% glycerol) and ground for 10 strokes. Each homogenate was transferred to a 2 ml Eppendorf tube and centrifuged at 10,000x g at 4°C for 10 min and the supernatant was reserved as the crude protein and stored at -80°C.

For preparation of mouse microsomal and cytosolic samples, to generate known metabolites for comparison, circa 1 g of mouse liver was homogenized in a 15 ml glass homogenizer containing 5 ml 0.1M ice-cold phosphate buffer (pH 7.4). The homogenates were filtered with four layers of cheesecloth and then spin at 10,000x g at 4°C for 10 min. The supernatant fraction was centrifuged at 100,000x g at 4°C for 1 hr to pellet liver microsomes and the resultant cytosolic supernatant was frozen in liquid nitrogen and stored at -80 °C.

2.3 Expression of insect P450s

Heterologous expression of *H. zea* P450s in Sf9 cells was carried out as described in Wen et al. (2003) and Sasabe et al. (2004) with minor modifications. The CYP321A1, CYP6B27 and CYP6B8 recombinant viruses were coexpressed with house fly P450 reductase recombinant virus at MOI (multiplicity of infection) ratios
of 2:2, 1:1 and 1:1, respectively, for 72 hr at 28°C. At the end of this incubation, 20 ml infected cells were pelleted by centrifugation at 5,000 rpm for 10 min and the cell pellets were washed once with an equal volume of 0.1 M phosphate buffer (pH 7.8) and resuspended in one-tenth volume cell lysis buffer (0.1 M phosphate buffer (pH 7.8), 1 mM EDTA, 0.5 mM PMSF, 0.1 mM DTT, 20% glycerol), sonicated two times for 30 sec (for 5 ml resuspended cells with a 30 sec rest between sonications). The sonicated cell lysates were cleared by centrifuging at 10,000 rpm for 10 min and the final supernatants were stored at -80°C. The P450 content of each sample was determined by CO difference analysis (Omura and Sato, 1964).

2.4 In vitro metabolism of AFB1

AFB1 metabolism assays conducted with midgut proteins were adapted from Lee and Campbell (2000) by extending reaction times to 3 hr, decreasing the temperature to 30°C and changing the pH of the phosphate buffer to 7.8. More specifically, each 250 µl reaction consisted of 2 mg/g tested crude protein [approximately 50 µl 10 mg/ml crude extract added in the reaction with the final concentration of 2 mg/ml], 0.1 mM phosphate buffer (pH 7.8), 0.5 mM NADPH, 0.5 mM GSH and 128 µM AFB1. The reactions were pre-incubated for 10 min at room temperature and initiated by addition of NADPH and GSH. After incubation at 30°C for 3 hr, the reactions were stopped by adding 1 ml cold methanol containing 10 µM AFG1 (as an internal control). The reaction mixtures were then centrifuged at 12,000x g for 10 min at room temperature and the supernatants were filtered on micro spin-down filter columns (Alltech, Deerfield, IL) before injection into the HPLC. Metabolites of AFB1 were analyzed by reverse-phase HPLC on a Supercosil LC-18 column (250 mm x 4.6 mm) equipped with a photodiode array UV detector using as mobile phase a mixture of water/methanol/acetonitrile (60/20/20) at a flow rate of 1 ml/min. In this system, the retention times for different standards were AFB1 (14 min), AFB2 (12.3 min), AFG1 (10.7 min), AFG2 (9.0 min) and AFM1 (7.5 min). AFB1 metabolites were identified by LC-MS analysis and comparison of their retention times with those of standards by HPLC chromatography. Due to limitations
in the number of larvae in our insect colony, we were unable to calculate steady-state kinetics for AFB1 metabolism in midgut homogenates.

AFB1 metabolism assays with P450s expressed in insect cells were set up as for midgut homogenates using varying amounts of sonicated Sf9 cell lysates containing 10 pmol CYP321A1, 25 pmol CYP6B8 or 25 pmol CYP6B27. The activities of CYP6B8, CYP6B27 and CYP321A1 were determined at final concentrations of 26 µM, 51 µM, 102 µM, 128 µM and 192 µM AFB1 in reactions terminated at 90 min with assays replicated three times for each independent biological sample. The disappearance rates of AFB1 by CYP321A1 in the reactions containing varying concentrations of AFB1 were calculated on the basis of substrate disappearance. Plots of AFB1 disappearance (Vs) relative to substrate concentration and the kinetic parameters (Vmax, Km) were made using Graphpad Prism version 3.03 software (Graphpad Software Inc., San Diego, CA).

PBO inhibition assays were conducted as above except that 1 mM PBO dissolved in N, N-dimethylformamide was added to reactions and these were compared to reactions containing an equal volume of N-dimethylformamide but lacking NADPH. Because there were no detectable metabolites using proteins isolated from control insects, crude proteins isolated from midguts of H. zea treated with coumarin or xanthotoxin were used for PBO inhibition studies.

2.5 Detection of AFBO conjugates

To trap AFBO conjugates, mouse liver cytosol (10 µl 50 mg/ml) or purified rat liver GSTs (50 µl/mg/ml) were added into the 250 µl reactions containing crude protein from H. zea midguts of larvae treated with coumarin or Sf9 cell lysates expressing CYP321A1. Reactions were stopped after incubation at 30ºC for 3 hr and 10 µl of filtered supernatant were injected for HPLC analysis.

2.6 LC-MS Analysis

AFB1 metabolites were collected from 20 metabolism reactions containing crude protein of H. zea induced with coumarin or 2 reactions conducted with 0.01 nmoles of heterologously expressed CYP321A1 protein. These metabolites
were vacuum-dried to a final volume of 0.5 ml and 50 µl of concentrated metabolites were collected for LC-MS analysis. LC/MS analysis was performed using the LCQ Deca XP electrospray ionization mass spectrometer equipped with a MS pump, autosampler and photodiode array detector (Thermo Fisher Scientific, Waltham MA).

2.7 RT-PCR Southern analysis

RNA was isolated from the homogenized midguts using TRIZol reagent (Gibco-BRL). One-step RT-PCR reactions were set up as described in Li et al. (2002b) with each 50 µl reaction including 100 ng RNA, 1x RT-PCR buffer, 20 U RNasin (Promega, Madison, WI), 5 U AMV reverse transcriptase (Promega), 10 pmole each forward and reverse primer and 1.25 U Taq polymerase (Invitrogen, Carlsbad, CA). The gene-specific forward/reverse primer sets used for CYP321A1 in these reactions corresponded to 5'-TAGTGTGGAGGGTGACCAACTG-3'/5'-CGGACAACAAGCCAGTCGTAGGC-3'. First stage RT reactions were incubated at 42ºC for 50 min and second stage PCR reactions proceeded with 18 cycles with each conducted at 94ºC for 1 min, 60ºC for 1 min and 72ºC for 1 min. Ten µl of each RT-PCR reaction were analyzed on 1% agarose-1x TBE gels and the RT-PCR products were transferred to Hybond-N membranes (Amersham-Pharmacia Biosciences, Uppsala) and hybridized with a 32P-labeled purified PCR fragment of the CYP321A1 cDNA at 42ºC in 5x SSC, 5x Denhardt's solution, 50% formamide, 50 mM sodium phosphate (pH 7.0) and 0.5% SDS. Membranes were washed twice for 15 min in low stringency buffer (2x SSC, 0.1% SDS) at 42ºC, twice for 15 min at high stringency buffer (0.2x SSC, 0.1% SDS) at 62ºC and quantified by Phosphorimager (Amersham-Pharmacia Biotech) analysis. RT-PCR Southern analyses were repeated at least two times with RNA samples prepared from midguts of larvae in two independent biological replicates.

2.8 Statistical analyses

All enzymatic reactions were repeated at least twice and the means of the replicates in different groups were compared and tested for significant differences from controls using the Tukey post-hoc test at $P=0.05$ with SAS software.

2.9 Docking of AFB1 in the CYP321A1 model
Development of the CYP321A1 and CYP6B8 models is discussed in detail in Rupasinghe et al. (2007). Briefly, seven eukaryotic or eukaryotic-like P450 crystal structures (CYP3A4 (1TQN) (Yano et al., 2004), CYP2C8 (1PQ2) (Schoch et al., 2004), CYP2B4 (1SUO) (Scott et al., 2004), CYP2C5 (1N6B) (Wester et al., 2003), CYP2C9 (1OG5) (Williams et al., 2003), CYP102 (2HPD) (Ravichandran et al., 1993), CYP2A6 (1Z1O) (Yano et al., 2005)) and one bacterial P450 crystal structure (CYP175A1 (1N97) (Yano et al., 2003)) were aligned using the ALIGN facility in MOE (Molecular Operating Environment) versions 2004 and 2005 (Chemical Computing Group Inc., Montreal, Canada) that uses the BLOSUM62 scoring matrix. The CYP6B8 and CYP321A1 sequences were then aligned to this fixed alignment and ten models were generated for each target sequence using the CYP3A4 crystal structure as the primary backbone template and coordinates from the CYP2B4 crystal structure for the highly divergent B’ helix B-C loop region and F-G loop region as detailed in Baudry et al. (2006). Docking of the AFB1 ligand was performed using LigandFit implemented in the program Cerius2 (Version 4.10. Accelrys, San Diego, USA). For this substrate, 100 possible conformations were generated and ranked according to dock score provided in LigandFit. The binding conformation for each protein with the highest score (lowest interaction energy) was selected as the optimal conformation and subjected to further energy minimization using the MMFF94 force field in MOE; heme coordinates were fixed to prevent distortion of the heme plane originating from bonded parameters in the MOE’s implementation of the MMFF94 force field.

3. Results

3.1 In vitro metabolism of AFB1 by *H. zea* midgut proteins

To determine the effect of plant allelochemicals on AFB1 metabolism, we compared AFB1 metabolism by *H. zea* using midgut proteins isolated from larvae fed on control diets and diets supplemented with plant allelochemicals (coumarin, xanthotoxin or indole 3-carbinol). While no metabolites were detected when AFB1 was incubated with midgut proteins isolated from larvae feeding on the control diet (Fig. 1A), the same two polar metabolites (Met1 with retention time of 7.0 min and
Met2 with retention time of 7.4 min) were readily detected when AFB1 (retention time of 14.0 min) was incubated with midgut proteins isolated from insects consuming diets containing coumarin or xanthotoxin (Fig. 1B and data not shown). Only trace amounts of a metabolite with the same retention time as Met1 were detected when AFB1 was incubated with midgut proteins isolated from insects consuming diets containing indole 3-carbinol (data not shown).

In \textit{in vitro} reactions using midgut proteins isolated from insects feeding on diets containing coumarin, production of the Met1 and Met2 metabolites required NADPH and was completely inhibited by PBO (piperonyl butoxide), a general P450 inhibitor, indicating that these derivatives were produced by P450s. LC-MS analyses of the AFB1 metabolites generated using midgut proteins isolated from coumarin-treated larvae indicate that Met1 has a molecular weight of 298, which is consistent with O-demethylation of AFB1 to produce AFP1 (Fig. 2), a hydroxylated metabolite of AFB1 by vertebrates which has been demonstrated to be less toxic than AFB1 (Stoloff et al., 1972; Eaton and Gallagher, 1994). Because it was recovered in such small quantities, we were unable to determine the molecular weight of Met2 using LC-MS analyses, but co-injection experiments conducted with aflatoxin M1 (AFM1, retention time 7.5 min) convincingly demonstrated that Met2 (7.4 min) is not this hydroxylated product.

Further analysis determined the turnover rates of AFB1 in reactions containing midgut proteins at a concentration of 2 mg/ml incubated at 30\(^\circ\)C for up to 3 hr. Under these conditions, the metabolites formed in reactions using midgut proteins isolated from coumarin-treated larvae increased linearly with time for at least 3 hr. Because commercial AFP1 was not available, AFB1 metabolism was evaluated both by the relative disappearance rate of the AFB1 parent compound as well as by the increased production of metabolites defined as the integrated metabolite areas divided by the integrated AFG1 (internal standard) area. As shown in Table 1, among all of the chemicals tested, coumarin is the most significant inducer of AFB1 metabolism in corn earworm with the disappearance rate for AFB1 being 51.4 pmol/min/mg protein and the appearance rates for Met1 and Met2 being
Xanthotoxin-induced midgut proteins also generated the same Met1 and Met2 metabolites but at significantly lower rates; in these samples, AFB1 disappearance was 27.0 pmol/min/mg protein and Met1 and Met2 production were nearly equivalent (10.9% and 9.4% of the AFG1 standard, respectively). The fact that the relative proportion of these metabolites differ in coumarin- and xanthotoxin-induced midgut samples indicates that more than one enzyme is involved in AFB1 metabolism in H. zea midguts.

3.2 In vitro metabolism of AFB1 by CYP321A1

To determine whether any of the known H. zea P450s are capable of metabolizing AFB1, CYP6B8 (Li et al., 2000), CYP6B27 (Li et al., 2002c) and CYP321A1 (Sasabe et al., 2004) were coexpressed with house fly P450 reductase using baculovirus expression systems as described in Wen et al. (2003) and Sasabe et al. (2004). Varying MOI ratios of recombinant P450 and P450 reductase viruses were tested to optimize activity of these P450s toward their common xanthotoxin substrate. After these optimizations, comparisons of the metabolic activities of CYP6B8, CYP6B27 and CYP321A1 toward AFB1 were conducted with cell lysates prepared from Sf9 cells coexpressing these proteins at MOI ratios of 1:1, 1:1 and 2:2 (P450: P450 reductase), respectively. No parent compound disappearance or metabolite formation was detected when AFB1 was incubated with either CYP6B8 or CYP6B27. In contrast, two metabolites with exactly the same retention time as Met1 and Met2 (Fig. 1) were detected when AFB1 was incubated with CYP321A1, suggesting that the AFB1 metabolites produced by CYP321A1 are the same as those formed by the corn earworm midgut proteins. The ratio of Met1 to Met2 generated in the reaction is around 1.8:1, which is lower than that obtained with midgut proteins of larvae induced with coumarin (2.8:1) and higher than that obtained with midgut proteins of the larvae induced with xanthotoxin (1.2:1) (Table 1). The AFB1 disappearance rate calculated in nmol AFB1/min/nmol P450 displays Michaelis-Menten kinetics (Fig. 3). Nonlinear regression analysis reveals that CYP321A1 metabolizes AFB1 with Vmax and Km values of 3.9 nmol AFB1 metabolized/min/nmol P450 and 241 µM,
3.3 Absence of AFBO-GSH conjugates

Given that AFB1 appears to be bioactivated into the more toxic AFBO, we determined whether H. zea larvae fed on diets containing coumarin were capable of converting AFB1 to AFBO. In vitro reactions conducted with midgut proteins induced with either of these treatments generated no detectable AFBO-conjugates even when mouse liver cytosol or purified rat liver glutathione S-transferases, which are both AFBO trapping agents, were added (data not shown).

3.4 Induction of CYP321A1 transcripts

The fact that H. zea larvae consuming control diets did not metabolize AFB1 at detectable levels and that both heterologously expressed CYP321A1 protein and midgut proteins isolated from larvae fed on phytochemical-containing diets generated the same two metabolites suggested that these phytochemicals might induce the CYP321A1 locus. Semi-quantitative RT-PCR gel blot analyses performed with RNA isolated from fifth instars fed for 48 hr on diets containing these phytochemicals (Fig. 4) indicate that all three plant allelochemicals tested (xanthotoxin, coumarin, indole-3-carbinol) are capable of inducing CYP321A1 transcripts compared with their low basal level in DMSO-treated control larvae. The magnitude of induction by these three phytochemicals corresponds to coumarin (42-fold), xanthotoxin (24-fold) and indole-3-carbinol (7-fold). This pattern of transcript inducibility at the CYP321A1 locus is consistent with the AFB1 metabolism profiles by midgut proteins isolated from larvae ingesting these phytochemicals: compounds failing to induce CYP321A1 also fail to induce AFB1 metabolism in the midguts of H. zea exposed to plant allelochemicals, and compounds inducing higher levels of CYP321A1 transcripts also induce higher levels of AFB1 metabolism in H. zea midguts.

3.5 Docking of AFB1 in the CYP321A1 model

To understand the binding modes for AFB1 in the predicted CYP321A1 and CYP6B8 catalytic sites, this CYP321A1 substrate was computationally docked in
both catalytic sites using the Monte-Carlo docking procedures within MOE and repeated cycles of protein and substrate minimization. Of 100 different conformations checked for this substrate in these two P450 sites, the lowest energy binding mode for each protein was subjected to further energy minimizations with all side chains fully relaxed. Examinations of the fully minimized AFB1 binding modes predict that it binds in the CYP321A1 catalytic site with the 8-methoxy group at a distance of 3.23 Å from the oxygen of the iron-oxo intermediate (Fig. 5) and in the CYP6B8v1 catalytic site at a distance of 7.63 Å. In these binding modes, AFB1 is predicted to have lower interaction energies for CYP321A1 (-50.0 kcal/mol) than for CYP6B8 (-41.0 kcal/mol). Among the side chains within AFB1 contact distance (<4.5 Å) shown in Fig. 5, several stand out as being remarkably conserved in these highly divergent P450s (aqua residues R104/R104 in SRS1, A305/A303 and E308/E306 in SRS4; each of these pairs is numbered with respect to CYP6B8/CYP321A1). Others are highly variable (elemental residues in Fig. 5) with some, such as F118/T119 and Q375/T373, contributing significantly to reducing the volume of the CYP6B8 catalytic site closest to the heme (180 Å³) compared to this same region in the CYP321A1 catalytic site (325 Å³). As a result, the methoxy group on AFB1 is predicted to dock substantially closer to the heme in the CYP321A1 model than to any region in the CYP6B8 model.

4. Discussion

Because metabolites of AFB1 were not detected in assays conducted with *H. zea* midgut proteins, it appears that this herbivore is not an efficient metabolizer of AFB1 or that it produces metabolites that are unstable. That AFB1 is more efficiently metabolized after exposure to plant allelochemicals is consistent with our previous finding that this polyphagous species is able to enhance its resistance to plant toxins and insecticides by increasing its P450-mediated metabolic capacities in response to certain allelochemicals (Li et al., 2000, 2002a, 2004; Sasabe et al., 2004). The further demonstration that CYP321A1 detoxifies AFB1 to the same two metabolites (AFP1 (Met1), Met2) generated by coumarin- and xanthotoxin-induced midgut proteins has
identified one significant allelochemical-inducible P450 responsible for protection against this fungal toxin. For CYP321A1, which shares many of the same catalytic activities as CYP6B8 (Sasabe et al., 2004; Rupasinghe et al., 2007), this represents a major innovation that can allow this insect to survive on food sources contaminated with *Aspergillus flavus* and *A. parasiticus*.

Inasmuch as the herbivorous *H. zea* likely never encounters mycotoxins in the absence of plant tissue, induction of AFB1 metabolism by co-occurring phytochemicals is consistent with the feeding ecology of this species. In vertebrates, multiple mechanisms are involved in phytochemical-induced chemoprotection against aflatoxins and related mycotoxins, including induction of phase I and phase II detoxification enzymes (P450s, GSTs, reductases) as well as the inhibition of P450s bioactivating aflatoxins (Kensler, 1997). Any one of the phytochemicals tested here may have multiple functions in *H. zea* as well: indole-3-carbinol is known to be capable of inducing rat P450s that activate AFB1 (CYP1A1, CYP1A2, CYP3A1, CYP3A2) as well as enzymes that detoxify AFB1 (aflatoxin B1-aldehyde reductase, glutathione S-transferase, NADPH:quinone oxidoreductase). The extent of chemoprotection in this vertebrate is determined by the balance attained between these bioactivation and detoxification processes (Stresser et al., 1994; Manson et al., 1998). Similarly, reduction of AFB1 toxicity by plant allelochemicals in *H. zea* may result from the involvement of CYP321A1 and other P450 and non-P450 detoxification systems.

The complete detoxification pathway for AFB1 in *H. zea* is not yet clear. In vertebrates, disposal of AFBO, a P450-mediated bioactivation product, represents an important detoxification pathway. Although the PBO inhibition assay of Zeng et al. (2006) provided evidence for the existence of AFB1 bioactivation processes in *H. zea* larvae that are P450-mediated, it is also possible that this compound, typically a P450 inhibitor, induces expression of detoxicative P450s, such as CYP321A1. This scenario is consistent with the AFB1 metabolic profiles characterized *in vitro* in two other insects, navel orangeworm (*Amyelois transitella*) and codling moth (*Cydia pomonella*) (Lee and Campbell, 2000). With more sensitive techniques used for
detection of AFB1 metabolites and more functional studies of *H. zea* P450s, future experiments will resolve these possibilities.

Providing further insight into our demonstration that CYP321A1 O-demethylates AFB1 to AFP1 while CYP6B8 does not, predictive AFB1-docked models derived for CYP321A1 and CYP6B8 suggest that the heme proximal region of the CYP321A1 catalytic site is large enough to allow AFB1 to approach within reactive distance of the iron-oxo intermediate. The significant reductions in the volume of this region predicted for the CYP6B8 catalytic site position the rigid AFB1 structure at a non-reactive distance from the heme oxygen. Among the many variable residues differentiating these catalytic sites, two bulky residues in CYP6B8 (F118 in SRS1, Q375 in SRS5) are the most likely contributors to the reduction in its catalytic site volume.

Since the discovery of the contributions of insect damage to aflatoxin contamination of crops in the early 1960s, attention has focused only recently on the competing mechanisms mediating aflatoxin detoxification and bioactivation in insects. Our identification of *H. zea* CYP321A1 as the major inducible P450 capable of detoxifying AFB1 to the more polar and less toxic AFP1 metabolite clarifies one mechanism by which phytochemicals enhance resistance to mycotoxins encountered by this insect in its fungus-contaminated hostplants.
References


Neunzig HH, 1963. Wild host plants of the corn earworm and the tobacco budworm


Table 1. Effects of plant allelochemicals on AFB1 metabolism by *Helicoverpa zea*.

<table>
<thead>
<tr>
<th>induction treatment</th>
<th>relative AFB1 metabolite generation (%)</th>
<th>AFB1 disappearance rate pmole/min/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Met 1</td>
<td>Met 2</td>
</tr>
<tr>
<td>control</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>coumarin</td>
<td>17.1 ± 0.7</td>
<td>6.1 ± 1</td>
</tr>
<tr>
<td>xanthotoxin</td>
<td>10.9 ± 1</td>
<td>9.4 ± 0.8</td>
</tr>
<tr>
<td>indole-3-carbinol</td>
<td>trace</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: not detected.
Fig. 1 HPLC chromatograms showing AFB1 metabolites generated by proteins isolated from midguts of *H. zea* fed on diets containing 1 mg/g coumarin. Metabolic reactions were carried out as described in Materials and Methods. Two metabolites, Met1 (retention time 7.0 min) and Met2 (retention time 7.4 min) were generated in the presence of NADPH (panel B) and not in its absence (panel A). AFG1 (retention time 10 min) represent the internal standard used for normalization of AFB1 remaining in each sample.
Fig. 2 LC-MS analysis of AFB1 metabolites. LC-MS analysis of metabolites showed that positive ionization of AFB1 metabolite Met1 (retention time 7.0) generates a compound with a molecular weight of 299.2. Deduction of a positive ion from this ionized metabolite results in a molecular weight of 298 which matches the molecular weight of O-demethylated AFB1 (aflatoxin P1).
**Fig. 3 CYP321A1 kinetics.** CYP321A1 coexpressed with house fly P450 reductase was assayed for AFB1 metabolism at varying substrate concentrations. The disappearance rate was calculated relative to P450 content in units of nmol AFB1 metabolized/min/nmol P450. The means with error bars represent the average of two biological replicates with each done in triplicate.
Fig. 4 Induction levels of CYP321A1 transcripts by different phytochemicals. 100 ng total RNA pooled from 20 midguts of newly molted fifth instar *H. zea* larvae reared on control diet or diets containing 0.2% DMSO, 1 μg/g AFB1 and phytochemicals (1 mg/g indole 3-carbinol, 1 mg/g coumarin, 0.5 mg/g xanthotoxin) for 48 hr were RT-PCR amplified with CYP321A1 and actin gene-specific primers. (A) The amplified products were Southern blotted and probed with 32P-labeled CYP321A1 and actin cDNA fragments as described in Materials and Methods and exposed and quantified by Phosphorimag. The CYP321A1 induction levels were calculated after normalization to the constitutive actin mRNA levels in each sample and are recorded relative to the negligible CYP321A1 levels in control larvae reared on a diet supplemented with only 0.2% DMSO. (B) The means of three independent RT-PCR amplifications are shown with error bars.
Fig. 5 AFB1-docked P450 models. Overlays of the AFB1-docked models built for the CYP321A1 and CYP6B8v1 proteins are shown with identical residues within contact distance (<4.5 Å) of AFB1 identified in aqua (R104/R104 in SRS1 is on the right, A305/A303 and E308/E306 in SRS4 are on the left) and variable residues in elemental colors. The two residues having highest impact on these active site volumes (F118/T119, Q375/T373) are labeled according to their identity in CYP6B8v1/CYP321A1. AFB1 is shown in CYP321A1 in orange ball-and-stick format and in CYP6B8v1 in purple ball-and-stick format.
Characterization of xenobiotic-metabolizing cytochrome P450s from *Amyelois transitella* (Walker) (navel orangeworm)

Abstract

The navel orangeworm *Amyelois transitella* (Walker) (NOW: Lepidoptera: Pyralidae) is a serious pest in California orchards and damages many crops including almonds, pistachios, walnuts and figs. To understand the molecular mechanisms underlying detoxification of phytochemicals, insecticides and mycotoxins by this species, three full-length cytochrome P450 monooxygenase cDNAs, CYP6AB11, CYP321C1 and CYP6B44, were isolated from larval midguts using Rapid Amplification of cDNA Ends PCR. Evolutionary relationships between these insect P450s and others with known functions were established by phylogenetic analysis. Metabolic assays conducted with baculovirus-expressed P450, P450 reductase and cytochrome *b*$_2$ samples and sixteen compounds, including phytochemicals, mycotoxins, and synthetic pesticides, showed that CYP6AB11 can efficiently metabolize imperatorin (0.88 pmol/min/pmol P450) and slowly metabolize piperonyl butoxide (0.11 pmol/min/pmol P450). LC-MS analysis indicated that the metabolite of imperatorin is the epoxide generated by oxidation of the double bond in its isoprenyl side chain. The predicted structure of CYP6AB11 suggests that its catalytic site contains a doughnut-like constriction over the heme that excludes aromatic rings on substrates and allows only their extended side chains to access the catalytic site. That CYP6AB11 can metabolize the principal insecticide synergist, piperonyl butoxide (PBO, a methylenedioxyphenyl synergist), in use to circumvent resistance raises the possibility that, once acquired, insecticide resistance in this species may be difficult to counter.
1. **Introduction**

Cytochrome P450 monooxygenases (P450s) comprise a superfamily of heme-thiolate enzymes in most aerobic organisms (Ortiz de Montellano, 2005). The reactions catalyzed by eukaryotic microsomal P450s are oxidations that release a water molecule through an electron-transport system involving cytochrome P450 reductase and cytochrome \( b_5 \). In insects, P450s metabolize many endogenous compounds critical for growth and development, including steroid hormones, juvenile hormones, and fatty acids (Feyereisen, 1999, 2006; Li et al., 2007). They are also important phase I detoxification enzymes converting a wide range of xenobiotics to more hydrophilic metabolites, which are excreted either directly or after conjugation to glucuronides or glutathiones mediated by phase II detoxification enzymes (Feyereisen, 1999, 2006; Li et al., 2007).

The many P450s in eukaryotes have been subdivided into 4 major clades (clans or subclasses) (Nelson, 1998; Feyereisen, 2006; Baldwin et al., 2009). Clade 3 in insects is further subdivided into the CYP6 and CYP9 families, which appear to participate primarily in xenobiotic metabolism (Feyereisen, 1999, 2006; Scott and Wen, 2001; Li et al., 2007). Within the genus *Papilio* (Lepidoptera: Papilionidae), CYP6 family members are known to detoxify furanocoumarins, secondary metabolites characteristic of the host plant families consumed by these insects (Cohen et al., 1992; Hung et al., 1995a, b, 1997; Wen et al., 2003, 2005, 2006a, b; Li et al, 2003; Pan et al., 2004). Across four *Papilio* species, close to a dozen P450s have been demonstrated to metabolize furanocoumarins; these include CYP6B1 and CYP6B3 from *P. polyxenes* (Cohen et al., 1992; Hung et al., 1995a, 1995b, 1997; Wen et al., 2003, 2005, 2006a, b; Pan et al., 2004), CYP6B4, CYP6B17 and CYP21 from *P. glaucus* (Li et al., 2002, 2003); CYP6B25 and CYP6B26 from *P. canadensis* (Li et al., 2003), and CYP6B33 from *P. multicaudatus* (Mao et al., 2007a, 2008a). Beyond the Papilionidae, other P450s in Clade 3 are known to metabolize furanocoumarins; these include CYP6AB3v1 and CYP6AB3v2 from the oecophorid *Depressaria pastinacella* (Mao et al., 2006b, 2007b, 2008b), which is a specialist on only two genera of furanocoumarin-containing plants, and CYP6B8 and CYP321A1
from *Helicoverpa zea* (Li et al., 2000, 2004; Sasabe et al., 2004; Rupasinghe et al., 2007), which is a polyphagous noctuid occasionally encountering furanocoumarins in a small number of its hostplants.

In general, across all lepidopteran families examined to date, P450 activity against furanocoumarins is correlated with the frequency with which these compounds are encountered in hostplants (Li et al., 2000, 2002, 2004, 2007; Mao, 2006a, b, 2007a, b, 2008a, b). CYP6AB3 from *D. pastinacella*, a specialist that feeds on reproductive structures of two furanocoumarin-containing host species, is the most specialized insect xenobiotic-metabolizing P450 yet characterized (Mao et al., 2006b, 2007b, 2008b). Of the many prospective substrates existing in its hostplants, this enzyme can metabolize only imperatorin, a linear furanocoumarin, and myristicin, a naturally occurring methylenedioxyphenyl (MDP) compound (Mao et al., 2006b, 2007b). By contrast, CYP6B8, a P450 identified from *Helicoverpa zea*, a highly polyphagous species recorded from over 100 plant species in many families, is able to metabolize many different structurally diverse classes of chemicals, including furanocoumarins, flavonoids and insecticides (Li et al., 2000, 2004; Rupasinghe et al., 2007).

The navel orangeworm *Amyelois transitella* is, like *H. zea*, a broadly polyphagous pest of a wide range of crop plants that in California orchards causes extensive damage to almonds, pistachios, walnuts, pomegranate, and figs (Connell, 2001; Campbell et al., 2003; Molyneux et al., 2007). In almond orchards, eggs are laid on mummy nuts on trees and larvae bore into nuts and eat their nutmeat until pupation. NOW also serves as a vector for the aflatoxin-releasing fungi *Aspergillus flavus* and *A. parasiticus* (Widstrom, 1979; Schatzki and Ong, 2000, 2001; Campbell et al., 2003) when wounds in fruits caused by NOW damage facilitate entry of spores. NOW infestations have often been linked with substantial aflatoxin contamination, rendering the crop unmarketable (Schatzki and Ong, 2000, 2001; Campbell et al., 2003). Demand for almonds worldwide is reaching unprecedented levels, leading to rapid expansion of almond acreage. Accordingly, to meet export requirements for aflatoxin contamination, minimizing NOW infestations is an important priority.
In comparison with most other insects, NOW is remarkably resistant to aflatoxin toxicity (Niu et al., 2009) due at least in part to its extremely active P450 detoxification system that can efficiently convert aflatoxin B1 (AFB1) into less toxic metabolites, including aflatoxin M1 (AFM1), aflatoxin B2a (AFB2a) and aflatoxicol (AFL) (Lee and Campbell, 2000). This active P450 detoxification system is likely also involved in allowing NOW to thrive on a broad range of chemically distinctive hostplants and may also serve as a preadaptation for acquiring resistance to synthetic organic insecticides. The absence of completely effective cultural or biological controls growers have remained heavily reliant on insecticides for NOW management. To better characterize the range of P450s contributing to xenobiotic metabolism in this species, we have cloned three full-length P450 cDNAs expressed constitutively in NOW larvae and expressed these in baculovirus-infected Sf9 insect cells. Their catalytic sites and predicted substrate binding modes have been predicted and compared with close relatives and with their observed metabolic activities.

2. Experimental procedures

2.1 Reagents

Four furanocoumarins (xanthotoxin, angelicin, bergapten, imperatorin), a coumarin (coumarin), a phenylpropanoid (myristicin), four flavonoids (quercetin, kaempferol, flavone, α-naphthoflavone), a phenolic acid (chlorogenic acid), a mycotoxin (AFB1, and two synthetic compounds (the insecticide α-cypermethrin, the synergist PBO) were used to test substrate specificity of heterologously expressed P450 proteins. All of these compounds and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma (St. Louis, MO). Three additional insecticides tested, aldrin, diazinon and methoprene, were bought from Indofine Chemical Company Inc. (San Francisco, CA). Reagents for heterologous expression in Sf9 insect cells, including Sf9 insect cells, SF-900 serum-free medium, and fetal bovine serum (FBS), were purchased from GibcoBRL/Life Technology (Grand Island, NY). All of the chemicals were dissolved in methanol and prepared as 10 mM stock solutions stored at -20°C. All HPLC solvents were bought from Fisher Scientific L.L.C. (Pittsburgh, PA, USA). House fly P450 reductase and fruit fly cytochrome b5
recombinant viruses were constructed by Dr. Zhimou Wen.

2.2 Insects

A laboratory colony of NOW was established and subsequently enriched with specimens generously provided by Dr. Joel Siegel from California. The colony was maintained in an insectary at University of Illinois at Urbana-Champaign at 28 ± 4°C with 16-h L/8-h D light cycle. NOW larvae were reared in 1000 ml plastic cups with small holes punched in the lid containing a wheat bran diet as described by Tebbets et al. (1978) and modified by Dr. Siegel (personal communication). NOW moths emerged approximately one month after the first instar stage. For breeding, around 20-30 adults were transferred to a clean 1000 ml plastic cup with a piece of tissue paper placed inside the cup and a piece of tissue paper covering the top.

2.3 Isolation of the full-length cDNA from *Amyelois transitella*.  

2.3.1 RNA isolation and 3’ RACE PCR amplification

Total RNA was extracted from 30 midguts dissected from larvae one day after molting to the final instar using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s directions. Superscript II (Invitrogen) was used to synthesize the first strand with C3PT as the oligo (dT)₁₇ primer (Table 1) and 1 µg of total RNA as the template. The first strand cDNA was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) to remove the primers and short cDNA; this product then became the 3’ RACE Ready DNA for use in the next RACE amplification.

A degenerate primer based on the conserved amino acid sequence of FDPER approximately 80 nucleotides upstream of the heme-binding region (Li et al., 2004) and a C3 adaptor primer (Table 1) were used to amplify putative 3’ cDNA sequences of P450 genes. The PCR reaction (50 µl) was heated to 94°C for 5 min, followed by 30 cycles of amplification (94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min) and followed by a final extension at 72°C for 10 min. After 1.5% agarose gel fractionation of the PCR products, DNA fragments in the size range of 300-700 bp were cut out, purified using a QIAquick gel extraction kit (Qiagen) and cloned into
the pGEM-T-easy vector (Invitrogen). Recombinant plasmids were isolated from 46 positive clones and their inserts were sequenced from both ends by the Core DNA Sequencing Facility at UIUC using vector primers. Among these clones, nine putative P450s fragments corresponding to three independent P450 sequences were identified by BLAST analysis.

2.3.2 5' RACE PCR

The 5’ ends of these three P450 sequences were amplified with the SMART™ RACE cDNA Amplification kit (Clontech, Mountain View, CA) following the manufacturer’s instructions. First-strand cDNA synthesis was performed using 1 µg total RNA, 1 µl CDS primer A and 1 µl SMART II A oligo and add sterile water to a final volume of 5 µl. After the mixture was incubated at 70°C for 2 min and cooled on ice for 2 min, 2 µl 5×First-strand buffer, 1µl 20 mM DDT, 1 µl 10 mM dNTP mix and 1 µl MMLV reverse transcriptase were added and the mixture was incubated at 42°C for 1.5 hr. After this incubation, the final mixture was diluted with the addition of 100 µl Tricine-EDTA buffer. and the 5’ RACE PCR amplification was set up by mixing 1 µl 10 mM of a gene-specific primer (62SM-GSP1, 34SM-GSP1 or 55SM-GSP1) (Table 1), 5 µl 10× Advantage PCR buffer, 1 µl 10 mM dNTP, 1 µl 50× Advantage 2 Polymerase Mix, 5ul 10× UPM primer stock and sterile water to a final volume of 50 µl. A PCR amplification reaction was set up sequentially with a program of 1 cycle at 94 °C for 5 min; 5 cycles at 94 °C for 30 sec, 72 °C for 3 min; 5 cycles at 94 °C for 30 sec, 70 °C for 30 sec, 72 °C for 3 min; 30 cycles at 94 °C for 30 sec, 68 °C for 30 sec, 72 °C for 3 min; the final extension at 72 °C for 10 min.

The reaction mixture was then diluted 50 times and 5 µl of the diluted PCR product was used to set up a nested PCR amplification with 1 µl 10 mM of a nested gene-specific primer (62SM-NGSP1, 34SM-NGSP1 or 55SM-NGSP1) and 5 µl 10×UPM primer stock. These reactions were PCR amplified with 1 cycle at 94 °C for 5 min; 25 cycles at 94 °C for 30 sec, 68°C (55SM-NGSP1 and 62SM-NGSP1) or 64°C (34SM-NGSP1) for 30 sec, 72 °C for 3 min; 1 cycle of final extension at 72 °C for 10 min. The PCR products were electrophoresed on a 1% agarose gel and those at
approximately 1.5 kb were excised and purified using a QIAquick gel extraction kit and then cloned into pGEM-T-easy vector. Plasmid DNAs isolated from white colonies on plates containing ampicillin, isopropyl β-D-1-thiogalactopyranoside (IPTG) and X-gal were sequenced at the Core DNA Sequencing Facility at UIUC.

2.3.3 Generation of the full length cDNA

The 3’ RACE-Ready cDNA described above was used as the template with a 5’ gene-specific primer (GN6AB11_CDS_forward, GN6B44_CDS_forward, or GN321C1_CDS_forward) corresponding to the start codon and a 3’ gene-specific primer (GN6AB11_CDS_reverse, GN6B44_CDS_reverse, or GN321C1_CDS_reverse) corresponding to the stop the codon was mixed with other PCR components including 2.5 µl 3’ RACE cDNA, 1 µl Advantage 2 polymerase, and 5 ul 10× Advantage PCR buffer, 1 µl dNTP (10 mM) and sterile water to a final volume of 50 µl. The PCR amplification was conducted with 1 cycle at 94°C for 5 min, 30 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 2 min and followed by a final cycle at 72°C for 10 min. The PCR products were electrophoresed on a 1% agarose gel and each approximately 1.5 kb fragment was excised and purified with a QIAquick PCR purification kit (Qiagen). To insert the PCR product into the pFastbac vector for sequencing and expression, the purified PCR product and pFastbac vector were digested with enzymes whose recognition sites were incorporated into their primers (Table 1) and purified with a QIAquick PCR purification kit. A ligation reaction was conducted in a reaction mixture containing 3 µl (150 ng) PCR product, 1 µl (100 ng) pFastbac, 1 µl T4 DNA ligase (Invitrogen), 1 µl 10×T4 DNA ligase buffer and 4 µl sterile water and incubated at 4°C overnight. The ligated DNA was transformed into Top10 competent cells (Invitrogen), plated on ampicillin plates and plasmids were isolated with QIAprep Spin Miniprep Kit (Qiagen) from the ampicillin-resistant colonies and sequenced at the Core DNA Sequencing Facility at UIUC.

2.4 Phylogenetic analysis

A phylogenetic analysis was performed for the 15 protein sequences selected to represent lepidopteran P450s involved in xenobiotics metabolism; these
included were CYP6B1 from *P. polyxenes* (black swallowtail), CYP321A1 and CYP6B8 from *H. zea* (corn earworm), CYP6B10 from tobacco budworm *Heliothis virescens* (tobacco budworm), CYP6B11 from *Papilio Canadensis* (Canadian tiger swallowtail), CYP6B12 from *P. glaucus* (Eastern tiger swallowtail), CYP6B29, CYP6AB4 and CYP6AB5 from *Bombyx mori* (domesticated silkworm), CYP321C1, CYP6AB11 and CYP6B44 from *A. transitella*, CYP6AB3v1 and CYP6B7 from *D. pastinacella* (parsnip webworm) and CYP321B1 from *H. armigera* (cotton bollworm). A phylogenetic tree was built using the neighbor-joining method in Mega 3.0 software and the inferred phylogeny was tested by bootstrap analysis with 1000 replicates (version 3.1, Kumar et al., 2004). The alignments, with amino acid substitutions labeled as strongly conservative, weakly conservative or nonconservative, were generated with ClustalW functions in SDSC Biology Software ([http://workbench.sdsc.edu](http://workbench.sdsc.edu)). The substrate recognition sites (SRS) regions in these proteins were determined by alignment with the CYP6B8 and CYP321A1 sequences described in Rupasinghe et al. (2007).

### 2.5 Baculovirus expression of *A. transitella* P450s

The CYP6AB11, CYP6B44 or CYP321C1 cDNA was introduced into the pFASTBac baculovirus expression vector as described in Niu et al. (2008) by transposing the pFASTBac clones containing the cDNA into DH10 competent cells and selecting on kanamycin/gentamicin/tetracycline/blue-gal/IPTG plates. Recombinant bacmid DNAs were isolated with a QIAprep Spin Miniprep kit (Qiagen), with the except of that, due to the large size of the bacmid DNA (>135kb), the isolated DNA was washed once with 70% alcohol instead of being column purified (as for smaller plasmids). To determine whether the P450 sequence was successfully inserted into the bacmid vector, PCR amplifications were conducted with an internal gene-specific primer (Table 1) and a M13 vector primer and the products were electrophoresed on 1% agarose gels. The recombinant viruses were generated by transfecting 1.5 μg (5μl) recombinant bacmid DNA containing the correctly sized insert into Sf9 cells using Cellfectin Reagent (Invitrogen) following manufacturer’s procedure. The recombinant baculoviruses were harvested after 72 hr
and amplified by infecting Sf9 cells with 500 µl of the recombinant virus stock and collecting the amplified virus particles after 48 hr. Plaque assays were used to determine the virus titer.

The P450 protein was co-expressed in Sf9 cells with two redox partners, house fly P450 reductase and fruit fly cytochrome b5, and the P450 content of each sample was determined by reduced CO difference analysis (Omura and Sato, 1964). In preliminary assays, the MOI (multiplicity of infection) ratios of the P450, P450 reductase and cytochrome b5 were varied to balance P450 production and activity. In the final assays, the MOI ratio (P450: P450 reductase: cytochrome b5) used for 1:1:0.1 (CYP6AB11), 1:1:0.1(CYP321C1) and 1:0.5:0.1 (CYP6B44). The infected cells were grown at 28°C and harvested after 72 hr with 10 µl 5 mg/g heme stock dissolved in 50% ethanol and 50% 1N NaOH added to each 10 ml culture plate 24 hr after the initial transfection. After this incubation, the virus particles were precipitated by centrifugation at 5,000 rpm for 10 min at 4°C and the cell pellets were washed once with an equal amount of 0.1 M phosphate buffer (pH 7.8) and resuspended in one-tenth volume cell lysis buffer [0.1 M phosphate buffer (pH 7.8), 1 mM EDTA, 0.5 mM PMSF, 0.1 mM DTT, 20% glycerol]. The resuspended pellets were sonicated for 15 sec per ml of protein pellet, centrifuged at 5,000 rpm for 10 min, and the final supernatant was frozen in liquid nitrogen and stored at -80°C prior to metabolism assays.

2.6 Metabolism assays by the heterologously expressed P450s

Metabolism assays were carried out in 0.5 ml reactions containing 0.1 M phosphate buffer (pH 7.8), 0.3 nmol P450 in cell lysis buffer and 10 nmol substrate. Reaction were initiated with the addition of 50 nmol NADPH and incubated at 30°C for 1 hr or longer with shaking and stopped with the addition of 100 µl of 1 M HCl. For the samples analyzed by either normal-phase HPLC or GC-MS, 400 µl of ethyl acetate including an internal standard (Table 2) were added to each reaction and the reaction mixtures were centrifuged at 10,000 rpm for 10 min at room temperature, after which 1 µl or 10 µl ethyl acetate extracted phase was injected for analysis. For the samples analyzed by reverse-phase HPLC, 0.5 ml acetone was added to each
reaction, samples were centrifuged at 10,000 rpm for 10 min and then 10 µl sample was injected into the reverse-phase HPLC for analysis. Details of the analytical methods are summarized in Table 2. The turnover rates of the parent compounds due to the activity of the P450 proteins were calculated based on substrate disappearance. Each metabolism assay was replicated three times.

2.7 Identification of the metabolite of imperatorin

The imperatorin metabolite generated by CYP6AB11 was analyzed by reverse phase HPLC to separate the metabolite from its parent compound. For this, a 200 µl aliquot of the ethyl acetate extracted phase from a 0.5 ml metabolism reaction containing 0.3 nmol P450 and 10 nmol imperatorin smf incubated for 1 hr at 30°C was dried under nitrogen and then dissolved in methanol. A 10 µl sample was injected onto the reverse-phase HPLC according to the method described in Table 2. After this, a 10 µl sample from the same reaction was examined by LC-MS (Shimadzu, model 2010EV, Kyoto, JP) using positive electrospray ionization (ESI) with the same solvent system but with a Luna C18 column (250 mm x 2 mm, 5 µm, Phenomenex).

2.8 Molecular modeling and substrates docking in the P450s

P450 structures were predicted using MOE programs (Chemical Computing Group Inc., Montreal, Canada) as previously described in Rupasinghe et al. (2003) and Mao et al. (2006b) using several templates, including CYP2C5 (1N6B; Wester et al., 2003), CYP2C8 (1PQ2; Schoch et al., 2004), CYP2C9 (1OG5; Williams et al., 2003), CYP3A4 (1TQN; Yano et al., 2004) and CYP102 (2HPD; Ravichandran et al., 1993). After energy minimization with CHARMm22 force field (MacKerell et al., 1998), each model was tested for its quality with Profiles 3D (Insight Homology Accelerys, CA, USA) and ProsaII (CAME Salzburg, Austria) and the best model was selected for substrate docking experiments.

The energy-minimized substrate-free protein structures were docked with substrate molecules, using the Monte-Carlo docking procedure of MOE with the MMFF94s force field (Halgren, 1996) for the oxygen-free heme as distributed in MOE 2004. The structures were initially placed above the heme plane and allowed to
vary through Monte-Carlo simulations. Hundreds of possible conformations were generated for each substrate inside the catalytic site and ranked according to the sum of the ligand's internal energy, van der Waals and electrostatic energy terms of the potential energy function. The lowest energy conformation was selected as the most feasible binding mode; the binding energy for each compound was calculated using the MMFF94s force field and the best-ranked binding modes for each compound were selected, included in the protein, and energy-minimized, assuring that the final energy gradient was less than 0.01 kcal/mol per Å. In the protein/ligand minimizations, the heme coordinates were kept fixed to avoid any distortion of the heme plane due to the lack of bonded parameters for the heme in the MMFF94s force field. Interaction energies between the minimized protein and the ligand were calculated as the difference between the total potential energy of the minimized complex and the sum of the individual protein and minimized components of the complex. Amino acids within a 4.5 Å radius around the substrate were selected as substrate contact residues and secondary structural elements containing these residues were selected as SRS.

3. Results

3.1 Gene analysis of P450s identified from A. transitella

To identify P450s involved in detoxification of phytochemicals, insecticides and mycotoxins, full-length P450 cDNAs from NOW larval midguts were amplified using 3’ RACE strategies with a degenerate primer corresponding to the conserved FDPER region in P450s approximately 30 amino acids upstream of the heme-binding region. These 3’ RACE clones were subsequently extended to their translation start sites using 5’ RACE strategies and gene-specific primers. The isolated P450 cDNAs classified as CYP6AB11 (516 amino acids), CYP321C1 (496 amino acids), CYP6B44 (502 amino acids).

Of more than 1800 P450 sequences identified from insects, those in the CYP6B and CYP6AB subfamilies and CYP321 family have been isolated exclusively from lepidopterans. Among these, 48 CYP6B genes are distributed across fourteen species, 12 CYP321 genes are distributed across seven species and 13
CYP6AB genes are distributed across five species (Table 2). Primary sequence alignments of these three protein sequences indicate that CYP6B44 shares 50-54% identity with other CYP6B proteins ranges, CYP321C1 shares 52% identity with CYP321A1 and 50% identity with CYP321B1, and CYP6AB11 shares 67% identity with CYP6AB4 (Fig. 1).

Comparisons of SRS regions in P450s with those in their close relatives indicate that amino acid identities in SRS4 (I helix) of these proteins are higher than in other SRS domains (SRS2, SRS3, SRS6), with up to 77% identity among all of selected P450s within each subfamily or family (Fig. 2). More specifically, comparisons of CYP6B44 and other CYP6B genes, 39 are absolutely conserved amino acids, 11 are strongly conserved, 11 are weakly conserved and 24 are not conserved (Fig. 2A). For CYP321C1 and CYP321A1 show that of the 84 amino acids in the SRS regions, of which 46 are absolutely conserved amino acids, 19 are strongly conserved, 8 are weakly conserved and 11 are not conserved (Fig. 2B). Of the 88 amino acids in the SRS regions of the CYP6AB proteins, 39 amino acids are absolutely conserved, 15 amino acids are strongly conserved, 3 amino acids are weakly conserved and 31 amino acids are not conserved (Fig. 2C).

3.2 Characterization of the substrates of the P450s from A. transitella

To characterize the substrates for the P450s isolated from NOW, the P450 cDNAs were co-expressed in Sf9 insect cells with house fly P450 reductase cDNA and fruit fly cytochrome b5 cDNA. Expression conditions were optimized by adjusting the ratio of P450: P450 reductase:cytochrome b5 to achieve maximum activity toward test substrates. The sixteen chemicals tested to determine their suitability as substrates were selected based on the predicted structures of these P450s, availability in recorded hostplants and analogy with similar P450s having known substrates. Among them, coumarin, xanthotoxin, angelicin, imperatorin and bergapten, flavone and α-naphoflavone have previously been determined to be metabolized by CYP6B proteins (Hung et al., 1997; Chen et al., 2002; Li et al., 2003, 2004; Wen et al., 2003, 2006b; Mao et al., 2007b) and H. zea CYP321A1 (Sasabe et al., 2004; Rupasinghe et al., 2007). Myristicin and imperatorin are the substrates for D.
pastinacella CYP6AB3 variants (Mao et al., 2006b, 2007b, 2008b). AFB1 has been frequently found in mummy almonds and pistachio housing NOW larvae and is now known to be substrate of CYP321A1 (Niu et al., 2008). Quercetin and kaempferol are flavonoids that are widely distributed in the hostplants of NOW and chlorogenic acid has been isolated from almonds and pistachios (Vaya and Mahmood, 2006; Rubilar et al., 2007; Bolling et al., 2009; Teets et al., 2009). Quercetin and chlorogenic acid are also efficiently metabolized by H. zea CYP6B8 (Li et al., 2004). In addition to natural products, several synthetic insecticides and their synergists were also tested to determine their suitability as P450 substrates, including α-cypermethrin, aldrin and diazinon and the insecticide synergist PBO. Of these, α-cypermethrin has been widely applied in orchards to control NOW (UC IPM online, 2009). Analysis of substrate disappearance in these assays (Table 4) indicated that imperatorin is efficiently metabolized by CYP6AB11 (0.88nmol/min/nmol P450) and PBO is turned over by CYP6AB11 at a slower rate (0.11nmol/min/nmol P450). CYP321C1 did not metabolize any of the 16 possible substrates tested in these assays. Only two compounds, xanthotoxin and aflatoxin B1, were tested with CYP6B44 but none of them can be metabolized.

3.3 Identification of the metabolite of imperatorin produced by CYP6AB11

Imperatorin disappearance detected by normal phase HPLC analysis was accompanied by the appearance of a new peak eluting at 3.3 min. To further separate the metabolite from its parent compound, samples were subsequently analyzed by reverse phase HPLC, where the metabolite of imperatorin generated in reactions containing NADPH eluted at the retention time of 16.1 min (Fig. 3B). This analysis indicated that the metabolite has a spectrum with maximum absorbance peaks at 299 nm, 249 nm and 213 nm, typical of the spectral characteristics of furanocoumarins (Fig. 3C).

LC-MS analysis was performed to further characterize the structure of the imperatorin metabolite. In positive mode electrospray, the metabolite yields four fragments: 203, 269, 287 and 309 (Fig. 4). Like the mass spectrum of imperatorin, the metabolite produces a fragment of mass equal to 202 (the plus 1 being 203 m/z),
which is equivalent to the fragment obtained from thermal degradation in GC-MS and is identical to xanthotoxol, the tricyclic structure with only an alcohol remaining from the isoprenyl side chain. The generation of the xanthotoxol fragment indicates that the aromatic rings are not the target of this enzyme. The fragment of mass 309 ion is adduct of the metabolite attached to a sodium ion and the intact metabolite appears to be an epoxide formed by the insertion of an oxygen atom into the double bond in the isoprenyl side chain. The fragment of mass 286 is equivalent to addition of exactly one atom of oxygen and the fragment of mass 287 is the +1 ion of this metabolite. The last fragment of mass 268 is an adduct attached to a sodium ion which may result from the loss of three carbons at the trailing end of the epoxide. This product is identical to that reported for CYP6AB3-mediated metabolism of imperatorin, which also appears to target the double bond in the isoprenyl side chain (Mao et al., 2006b, 2007b). No metabolite of PBO was observed in our GC-MS analysis.

3.4 Structure predictions and substrate docking

Alignment of CYP6AB11 with the available mammalian P450 templates indicated that the template with the highest sequence identity for CYP6AB11 was CYP3A4 (31% sequence identity). The CYP3A4 template was, in fact, used to build the original *D. pastinacella* CYP6AB3 model (Mao et al., 2006b) and we used it as the template to predict the CYP6AB11 structure. At the level of primary sequence alignments, CYP6AB11 is 55% identical to CYP6AB3 and the molecular models of both proteins are structurally similar. Overlays of their active sites (Fig. 5) indicate that most of the active site residues are conserved, particularly those close to the heme, where they form a doughnut-like structure similar to that described in the CYP6AB3 molecular model (Mao et al. 2006b). Even so, CYP6AB11 and CYP6AB3 do not exactly align since there are amino acid insertions of Pro105 (in SRS1) and Pro235 (between SRS2 and SRS3) in CYP6AB3. Interestingly, all of the amino acids creating the doughnut-like structure constricting the catalytic site are conserved except for Ile310 (CYP6AB3) vs. Val308 (CYP6AB11) (Fig. 5). Docking of imperatorin in the active site predicts that imperatorin binds in the same orientation
as in CYP6AB3 but with a slightly higher interaction energy of 38.4 kcal/mol and at a slightly greater distance from the heme estimated to be 7.2 Å. The Ile310-to-Val308 replacement in CYP6AB11 enlarges the opening to the heme and is predicted to allow imeratorin to be slightly more mobile than in the CYP6AB3 catalytic site.

4. Discussion

Without a fully sequenced genome, characterizing the xenobiotic-metabolizing P450 inventory of a particular species is experimentally challenging. Our effort to identify the subset of P450s in the NOW genome associated with xenobiotic metabolism, however, was greatly facilitated by the use of bioinformatics, homology modeling, and comparative ecology, which allowed us to build on prior work identifying substrate specificities in related P450s. Thus, we were able to demonstrate that CYP6AB11 is able to metabolize imeratorin at a significant rate (0.88 pmol/min/pmol P450). Imectorin is a toxic furanocoumarin that has been isolated from a range of plant species in the family Apiaceae (Berenbaum, 1991; Berenbaum and Zangerl, 1998; Baek et al., 2000). While NOW has no known apiaceous hostplants, it is commonly found on fig (Ficus carica), a moraceous plant reported to contain several linear furanocoumarins, including both oxypeucedanin and oxypeucedanin hydrate, which, like imectorin, contain an 8-O-prenylated side chain (Zaynoun et al., 1984; Towers, 1986). In other species, furanocoumarins with an extended side chain at the 8 position are metabolized by different P450s from those which metabolize 8- or 5-methoxy-substituted furanocoumarins. For example, whereas CYP6B1 in P. polyxenes, the black swallowtail, metabolizes both xanthotoxin (8-methoxypsoralen) and bergapten (5-methoxypsoralen) at high rates, it apparently has little to no catalytic activity against imectorin (Cohen et al. 1992). Conversely, CYP6AB3 from D. pastinacella, which is highly active against imectorin, has little to no catalytic activity against either bergapten or xanthotoxin (Mao et al., 2006b, 2007b). Among lepidopteran furanocoumarin-metabolizing P450s, only CYP6B4 from the polyphagous P. glaucus can metabolizes imectorin as well as 5-methoxy substituted furanocoumarins, possibly because several amino acid differences in substrate recognition sites enlarge the catalytic pocket to accommodate
a greater range of substrates (Li et al., 2003).

CYP6AB11 shares 55% identity with CYP6AB3 and, based on a comparison of their predicted 3-dimensional structures, also shares a doughnut-like structure immediately above the heme that limits substrate access to the catalytic site. Key residues limiting access include Phe118 in SRS1 and Ala314 and Thr318 in SRS4 and Leu380 in SRS5, which are conserved both in CYP6AB3 and CYP6AB11 (Fig. 5) (Mao et al., 2006b). This doughnut structure in CYP6AB11 and CYP6AB3 has not been found in other CYP6 family proteins sequenced to date. The imperatorin metabolite generated by CYP6AB11 possesses an epoxide side chain, the result of attachment of oxygen to the side chain double bond; the CYP6AB11 model docked with imperatorin shows that this substrate can bind in the same orientation as it does in CYP6AB3. Comparison of the predicted interaction energies shows that in imperatorin would bind in the CYP6AB11 catalytic site with a slightly higher interaction energy consistent with the metabolic assay results. The protein alignment within the CYP6AB families reveals that CYP6AB11 does not have an Ala92Val replacement that characterizes the CYP6AB3v2 variant, which is predicted to greatly increase imperatorin metabolism by increasing electron transfer between P450 and P450 reductase (Mao et al., 2007b). The absence of this replacement helps to explain why the catalytic activity of CYP6AB11 is closer to that of CYP6AB3v1 than that of CYP6AB3v2.

PBO is known as a general P450 inhibitor and has historically been used as a synergist applied with pyrethroid insecticides to control insects. It has two rings plus a long side chain (C₉H₁₉O₃) as well as a short side chain (C₃H₇). Mechanisms of PBO metabolism are complex and not fully clear; most studies were completed with mammalian P450s have identified more than 10 metabolites, but the specific biochemical pathways leading to their formation have not yet been fully elucidated (Byard and Needham, 2006). Although CYP6AB11 exhibits low levels of activity toward PBO, we were unable to detect a metabolite by GC-MS. Surprisingly, CYP6AB11 displayed no consistent detectable activity against myristicin, another MDP compound that shares both a core structure and synergistic activity with PBO;
myristicin levels declined less than 5% in the metabolism reactions incubated for 2 hr. In contrast, CYP6AB3v2 actively metabolizes myristicin (Mao et al., 2008b). Because the disappearance rate is close to the detection limit and because the disappearance rate is highly variable across replicates, it is still not clear whether myristicin can be metabolized by CYP6AB11.

Although CYP321A1 from *H. zea* displays activity against a very broad range of phytochemical, insecticide and mycotoxin substrates (Sasabe et al., 2004; Rupasinghe et al., 2007; Niu et al., 2008), using similar techniques I was unable to demonstrate metabolic activity of CYP321C1 against any of the 16 tested chemicals. The protein alignment of CYP321C1 with CYP321A1 shows that there is a high level of amino acid identity at the back of the catalytic site in the I helix (SRS4) and the loop sequences above the catalytic site (SRS3), but there are also many divergent amino acid residues in other SRS regions which might be critical for substrate channel architecture. Our docking models show that CYP321A1 has a spacious cavity allowing larger and more rigid molecules, such as aflatoxin B1, to access its catalytic sites. Compared with the CYP321A1 molecular model, CYP321C1 has a smaller catalytic core structure which may block access to the catalytic sites for larger molecules (data not shown).

Only two compounds, xanthotoxin and aflatoxin B1, were examined as possible substrates for CYP6B44, while previous work with *P. polyxenes* CYP6B1 suggested some degree of specialization for furanocoumarin metabolism. No metabolism of either compound could be detected with CYP6B44. Amino acids critical for metabolism of furanocoumarins in the SRS domains of CYP6B proteins include Phe116 and His117 in SRS1, Phe371 in SRS5 and Phe484 in SRS6, which are predicted to form a network that stabilizes the catalytic sites and allows the furanocoumarins to reach its catalytic core (Chen et al., 2002; Baudry et al., 2003). Among them, Phe116 in SRS1 is conserved in all CYP6B proteins and His117 is conserved except in CYP6B44 where Asn117 replaces His117. The other amino acids including Phe371 in SRS5 and Phe484 in SRS6 are divergent between different CYP6B proteins, which may contribute in part to substrate specificity (Fig. 2A).
Substitution of Ile115 for Leu115 in SRS1 of CYP6B1 can greatly enhance its activity via changes in the metabolite release channel (Pan et al., 2004; Wen et al., 2005). The fact that the Ile115Leu replacement is found in CYP6B44 should facilitate furanocoumarin metabolism. CYP6B8 can metabolize more diverse chemicals than the specialized furanocoumarin metabolizing P450s. The molecular model built by Li et al. (2004b) shows that CYP6B8 has a larger and more flexible catalytic site than CYP6B1 and also has one more substrate access channel than CYP6B1.

The presence of a seemingly fairly specialized CYP6B11 in the broadly polyphagous NOW appears to contrast with previous findings that CYP6B genes in polyphagous lepidopterans are as a rule broadly substrate-specific. Our effort to identify more substrates by subjecting a whole-plant extract to metabolism assays (cf., Mao et al., 2008b) failed to reveal any additional suitable substrates, despite considerable chemical complexity present in these extracts. This seemingly specialized enzyme may be a historical vestige of a closer association with a particular subset of hostplants. Navel orangeworms, as the name suggests, were originally described from Citrus spp. (Mote, 1922), although such host use is rarely reported today (Mahoney et al., 1989). It is intriguing to note, however, that citrus and other rutaceous species are rich in furanocoumarins and may have at some point in the evolution of this species represented a major host and selective agent on metabolic detoxification pathways. An examination of the host use patterns and detoxification capacity of the five congeners of A. transitella may shed light on the significance of furanocoumarins in the evolution of this pest species.
References


Li W, Zangerl AR, Schuler MA, Berenbaum MR, 2004a. Characterization and


Mao W, Zangerl AR, Berenbaum MR, Schuler MA, 2008b. Metabolism of myristicin


Table 1 Primers used for 5’ and 3’ RACE amplification of P450s from *Amyelois transitella*

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’ to 3’)</th>
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<tr>
<td>FDPER primer</td>
<td>GCCGATCCTT(T/C)GA(T/C)CC(I/A)AG(A/G)AG(A/G)TT</td>
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</tr>
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<tr>
<td>GN66B44-CDS_forward</td>
<td>GGTCTAGACTGAGATGATCCTTTTTATTTTTGTTGCTAATAAC</td>
</tr>
<tr>
<td></td>
<td>Xba I</td>
</tr>
<tr>
<td></td>
<td>Pst I</td>
</tr>
<tr>
<td>GN66B44_CDS_reverse</td>
<td>GGCTCGAGAAGCTTTTTATACCTAGGTTGAACCTTCAAATGC</td>
</tr>
<tr>
<td></td>
<td>Xho I</td>
</tr>
<tr>
<td></td>
<td>Hind III</td>
</tr>
<tr>
<td>GN321C1-CDS_forward</td>
<td>GGTCTAGAATGTATTGTTCCAGATAATCTGTTAATTATTTATG</td>
</tr>
<tr>
<td></td>
<td>Xba I</td>
</tr>
<tr>
<td>GN321C1_CDS_reverse</td>
<td>GGCTCGAGAAGCTTTTTATATGTATTCTCCCCAAATGATTCAATAC</td>
</tr>
<tr>
<td></td>
<td>Xho I</td>
</tr>
<tr>
<td></td>
<td>Hind III</td>
</tr>
</tbody>
</table>
Table 2 Analytical methods for the substrates of the metabolism assays.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Internal standard</th>
<th>Analysis method</th>
<th>Elution solvent</th>
<th>Detection (nm)</th>
<th>Elution time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>xanthotoxin</td>
<td>bergapten</td>
<td>Normal HPLC</td>
<td>80% cyclohexane, 18% diethyl ether and 2% butanol; 1.5ml/min flow rate</td>
<td>254</td>
<td>5.1</td>
</tr>
<tr>
<td>bergapten</td>
<td>xanthotoxin</td>
<td>Normal HPLC</td>
<td>80% cyclohexane, 18% diethyl ether and 2% butanol; 1.5ml/min flow rate</td>
<td>254</td>
<td>4.2</td>
</tr>
<tr>
<td>angelicin</td>
<td>xanthotoxin</td>
<td>Normal HPLC</td>
<td>80% cyclohexane, 18% diethyl ether and 2% butanol; 1.5ml/min flow rate</td>
<td>254</td>
<td>3.1</td>
</tr>
<tr>
<td>coumarin</td>
<td>xanthotoxin</td>
<td>Normal HPLC</td>
<td>80% cyclohexane, 18% diethyl ether and 2% butanol; 1.5ml/min flow rate</td>
<td>254</td>
<td>3.5</td>
</tr>
<tr>
<td>imperatorin</td>
<td>xanthotoxin</td>
<td>Normal HPLC</td>
<td>80% cyclohexane, 19% diethyl ether and 2% butanol; 1.5ml/min flow rate</td>
<td>254</td>
<td>3.8</td>
</tr>
<tr>
<td>flavone</td>
<td>xanthotoxin</td>
<td>Normal HPLC</td>
<td>80% cyclohexane, 18% diethyl ether and 2% butanol; 1.5ml/min flow rate</td>
<td>254</td>
<td>3.9</td>
</tr>
<tr>
<td>α-nathoflavone</td>
<td>xanthotoxin</td>
<td>Normal HPLC</td>
<td>80% cyclohexane, 18% diethyl ether and 2% butanol; 1.5ml/min flow rate</td>
<td>254</td>
<td>3.0</td>
</tr>
<tr>
<td>quercetin</td>
<td>None</td>
<td>Reverse HPLC</td>
<td>45% methanol, 55% water in 0.2% acetic acid; 1ml/min flow rate</td>
<td>375</td>
<td>20.1</td>
</tr>
<tr>
<td>kamperol</td>
<td>None</td>
<td>Reverse HPLC</td>
<td>60% methanol, 40% water in 0.2% acetic acid</td>
<td>375</td>
<td>8.9</td>
</tr>
<tr>
<td>chlorogenic acid</td>
<td>None</td>
<td>Reverse HPLC</td>
<td>7% acetonitrile, 93% water in 0.01% formic acid; 1ml/min flow rate</td>
<td>328</td>
<td>22.1</td>
</tr>
<tr>
<td>imperatorin</td>
<td>xanthotoxin</td>
<td>Reverse HPLC</td>
<td>Gradient solvent system: pump A Water in 0.05% formic acid; B methanol; Start from 80%A for 2 min; condition the column from 80%A to 100%B in 13min; remain in 100%B for 5 min; condition the column from 100%B to 80%A for 2 min; remain in 80%A for 10 min.</td>
<td>254</td>
<td>19.6</td>
</tr>
<tr>
<td>myristicin</td>
<td>methoprene</td>
<td>GC-MS</td>
<td>In the legend.</td>
<td></td>
<td>14.1</td>
</tr>
<tr>
<td>PBO</td>
<td>methoprene</td>
<td>GC-MS</td>
<td>In the legend.</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td>alpha-cypermethrin</td>
<td>methoprene</td>
<td>GC-MS</td>
<td>In the legend.</td>
<td></td>
<td>32.4</td>
</tr>
<tr>
<td>aldrin</td>
<td>methoprene</td>
<td>GC-MS</td>
<td>In the legend.</td>
<td></td>
<td>19.5</td>
</tr>
<tr>
<td>diazinon</td>
<td>methoprene</td>
<td>GC-MS</td>
<td>In the legend.</td>
<td></td>
<td>16.7</td>
</tr>
<tr>
<td>aflatoxin B1</td>
<td>aflatoxin</td>
<td>Reverse HPLC</td>
<td>60% water, 20% acetonitrile;20% methanol; 1ml/min flow rate</td>
<td></td>
<td>14.5</td>
</tr>
</tbody>
</table>

1 Reverse-phase HPLC system consists of Waters 501 pump, Waters WISPC autosampler and Waters 996 photodiode array (PDA) and Supercosil LC-18 column (250 mm × 4.6 mm).
2 Normal phase HPLC system consist of Waters WISP 710B autosampler, Waters M-45 solvent delivery system, Waters 440 absorbance detector, Hewlett Packard 3390A integrator and Waters silica column (4.6*50 µm).

3 GC-MS system: GC-2010 chromatograph spectrometer, GCMS-QP2010 plus mass spectrometer, AOC-20S Shimadzu autosampler and column (Shimadza, Kyoto, JP). GC-MS program: injection temperature 290 °C, column temperature starts from 40°C, hold for 1 min, 30 °C/min to 130 °C, at 5 C/min to 250 °C, at 10°C/min to 300°C, hold for 5 min; ion source temperature 230 °C, interface temperature 280°C. The purified helium flow for GC-MS is 1.5 ml/min. The area of the chemical was integrated based on total ion chromagraph profile by the software with the system.
Table 3 Distribution of individual genes from CYP6B and CYP6AB and subfamily CYP321 in Lepidopterans (from http://drnelson.utmem.edu/cytochromeP450.html).

<table>
<thead>
<tr>
<th>Insect Species</th>
<th>CYP6B</th>
<th>CYP6AB</th>
<th>CYP321</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Papilio polyxenes</em></td>
<td>1,3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Helicoverpa zea</em></td>
<td>2, 8,9, 27</td>
<td>9, 10</td>
<td>A1, A2</td>
</tr>
<tr>
<td><em>Helicoverpa armigera</em></td>
<td>6,7</td>
<td>9, 10</td>
<td>A1, A3, A4, A5, A6, B1,</td>
</tr>
<tr>
<td><em>Heliothis virescens</em></td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Papilio canadensis</em></td>
<td>11, 13, 14,15, 18,</td>
<td>4, 5, 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19,22, 25, 26</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Papilio glaucus</em></td>
<td>12, 16, 17, 20, 21,</td>
<td>4, 5, 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>23,24</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bombyx mori</em></td>
<td>29</td>
<td>4, 5, 8</td>
<td></td>
</tr>
<tr>
<td><em>Papilio multicaudatus</em></td>
<td>30, 32, 33, 34, 35, 36,</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Spodoptera exigua</em></td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Spodoptera frugiperda</em></td>
<td>38, 39, 40, 41, 42</td>
<td>A7, A8, A9</td>
<td></td>
</tr>
<tr>
<td><em>Helicoverpa armigera</em></td>
<td>43</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Amyelois transitella</em></td>
<td>44</td>
<td></td>
<td>C1</td>
</tr>
<tr>
<td><em>Manduca sexta</em></td>
<td>45, 46</td>
<td>13</td>
<td>D1</td>
</tr>
<tr>
<td><em>Spodoptera littura</em></td>
<td>47, 48</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td><em>Trichoplusia ni</em></td>
<td>1, 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Depressaria pastinacella</em></td>
<td>3, 7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Number represents the name of the individual P450 in the subfamily.
Table 4 Metabolism rates by CYP6AB11.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
<th>Metabolism Rate (nmol/min/nmol P450)</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imperatorin</td>
<td><img src="image1" alt="Imperatorin structure" /></td>
<td>0.88</td>
<td>0.11</td>
</tr>
<tr>
<td>Piperonyl butoxide</td>
<td><img src="image2" alt="Piperonyl butoxide structure" /></td>
<td>0.11</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Fig. 1. The phylogenetic tree of the genes from CYP6AB, CYP6B and CYP321 in insects. The genes tested were from the different insect species (Table 1). The rooted phylogenetic tree was constructed using the neighbor-joining method built in Mega 3.1 and the inferred phylogeny was tested by bootstrap analysis with 500 replicates. The branch lengths are proportional to p-distance with numbers representing the bootstrap calculated.
Fig. 2 The protein alignment with the labeled SRS domains of the P450s from insects including CYP6AB (Fig. 2A), CYP6B(Fig. 2B) and CYP321(Fig. 2C) subfamilies. Fig. 2A The sequences of CYP6B1 (Papilio polyxenes), CYP6B8 and CYP321C1 (Helicoverpa zea); CYP6B4 (Papilio glaucus), CYP6B44, CYP321C1 and CYP6B11v1 (Amyelois tansitella); CYP6AB3v1 and CYP6AB3v2 (Depressaria pastinacella) were found from the NCBI protein data base. SRS indicates the substrate recognition sites and the amino acids in gray are within SRS regions. Consensus key symbols as follows: “*”single, fully conserved residue; “:”conservation of strong groups; “.”conservation of weak groups; “no label”
represents no consensus.

Fig. 2A (cont.)
Fig. 2B The protein alignment with the labeled SRS domains of the P450s from insects including CYP321 families.
Fig. 2C The protein alignment with the labeled SRS domains of the P450s from insects including CYP6AB subfamilies.
Fig. 2C (cont.)
Fig. 3. Reverse-Phase HPLC chromatogram showing imperatorin metabolite produced by heterologously expressed CYP6AB11 in insect cells. Metabolic reactions were carried out as described in Materials and Methods. The metabolite of imperatorin was eluted at 16.1 min in the reaction with addition of NADPH (B) compared with the reaction without addition of NAPDH (A). Xanthotoxin as internal standard was eluted at 16.6 min. The imperatorin metabolite was scanned with the HPLC photodiode array detector and the spectrum of the imperatorin was shown in Fig. 3C.
Fig. 4. LC-MS spectrum of the imperatorin metabolite by CYP6AB11.
Fig. 5. Predicted imperatorin binding in CYP6AB11 and CYP6AB3 models. Residues around a 4.5 Å radius are shown. Conserved residues are shown in green while non-conserved residues are shown in elemental colors. Imperatorin binding conformation in CYP6AB3 is shown in aqua and the conformation in CYP6AB11 is shown in orange.
VI Effects of naturally occurring and synthetic synergists on the toxicity of insecticides, phytochemicals and mycotoxins to *Amyelois transitella* (Lepidoptera: Pyralidae)

Abstract

The navel orangeworm (NOW), *Amyelois transitella*, is among the most destructive pests of almonds in California as well as a serious problem in pistachios (*Pistacia vera*), figs (*Ficus carica*) and walnuts (*Juglans regia*). NOW feeding leaves the plants vulnerable to infection by *Aspergillus* spp., fungi that produce toxic aflatoxins. The primary aflatoxin of concern to regulators is aflatoxin B1 (AFB1). Due to the extreme carcinogenicity of these mycotoxins, acceptable aflatoxin levels in foods in the European Union have been set very low and present a challenge to the California tree nut industry. A potentially safe and sustainable approach for managing NOW in almond orchards may be to use natural essential oil synergists to interfere with this insect’s ability to detoxify mycotoxins, phytochemicals and insecticides. We tested the effects of a naturally occurring plant-derived chemical, myristicin (MYR), and a synthetic inhibitor of cytochrome P450 monoxygenases (P450s), piperonyl butoxide (PBO), on the toxicity of three insecticides (α-cypermethrin, τ-fluvalinate and methoxyfenozide), a phytochemical (xanthotoxin) and a mycotoxin (AFB1) to *A. transitella*. The results of the insecticide assays show that α-cypermethrin and methoxyfenozide are more toxic than τ-fluvalinate to the newly hatched larvae (56-fold and 9.6-fold at 48 hr, respectively). The PBO and myristicin synergism bioassays with insecticides show that PBO significantly synergizes cypermethrin and fluvalinate and increases their toxicity to NOW, while myristicin synergizes only cypermethrin. Neither compound has any significant synergistic effects on methoxyfenozide. Synergism bioassays with xanthotoxin demonstrate that PBO significantly synergizes the toxicity of this phytochemical over time and that myristicin only slightly increases mortality after 7 days. None of the potential synergists tested enhanced AFB1 toxicity to NOW larvae. In view of these findings and the limited availability of environmentally safe synthetic insecticides for sustainable management of NOW, myristicin should be explored as a field treatment.
to reduce survival of this pest species and aflatoxin contamination in orchard situations.

1. Introduction

The navel orangeworm (NOW), *Amyelois transitella*, is among the most destructive pests of almonds in California, as well as a serious problem in pistachios, figs and walnuts. Neonates tunnel into the nut and successive instars consume the nutmeat, generating large quantities of frass and webbing (Bentley et al. 2008). In addition to causing such direct losses, NOW feeding leaves almonds vulnerable to infection by *Aspergillus* spp. that produce toxic aflatoxins (Campbell et al., 2003). NOW adults lay eggs in mummy fruits when new crop nuts are unavailable (Connell, 2001; Molyneux et al., 2007) and the caterpillars have been reported as scavengers on mummified fruits or nuts of at least 25 plant species (Shelton and Davis, 1994).

Aflatoxins, considered to be potent hepatocarcinogens, have become a serious health issue in many countries, especially in Asia and Africa. As a result, strict regulations have been applied to reduce aflatoxin levels in foods (Williams et al., 2004; Wagacha and Muthomi, 2008). The Food and Drug Administration (FDA) in the United States set a maximum 20 ppb limit for total aflatoxins in food, including edible nuts, and a maximum 0.5 ppb in milk (Food and Drug Administration, 2006). The European Union, one of the principal export targets of the California nut industry, sets even lower limits: nuts can contain a maximum level of 8 μg/kg (ppb) aflatoxin B1 (AFB1) and 10 μg/kg (ppb) of total aflatoxins (http://gain.fas.usda.gov/Recent%20GAIN%20Publications/New%20EU%20Aflatoxin%20Levels%20and%20Sampling%20Plan_Brussels%20USEU_EU-27_3-9-2010.pdf). For milk, the maximum level is 0.05μg/kg (ppb) aflatoxin M1 (AFM1) (Molyneux et al., 2007) These strict limits have resulted in an increasing frequency of rejections of U.S. nuts by the EU due to aflatoxin contamination between 2005 and 2009; in 2005 alone, 41 rapid alert and information notifications were issued to the U.S. (Molyneux et al., 2007). Aflatoxin contamination is seriously challenging the California tree nut industry.

Fungal contamination in orchards is frequently reported to be associated
with NOW damage and the control of NOW decreases aflatoxin levels in crops (Widstrom, 1979; Schatzki and Ong, 2000, 2001; Campbell et al., 2003). Currently, NOW management depends on a combination of control tactics, including cultural practices and insecticide sprays, particularly when infested adjacent crops, such as pistachios, provide immigrants into almond orchards (Higbee and Siegel, 2009). Biological control agents, although identified, cannot yet provide complete control. Chemical sprays for hull splits include organophosphates and pyrethroids; at present, only Entrust (spinosad) sprays are acceptable in organic orchards (Campbell et al., 2003; Molyneux et al., 2007). Recent studies have demonstrated that *A. transitella* tolerates very high levels of aflatoxin (>100 µg/g) in its diet (Niu et al., 2009) due to the activity of detoxification enzymes, including cytochrome P450 monooxygenases (P450s) (Lee and Campbell, 2000; Niu et al., 2009). This dependence on P450s for aflatoxin tolerance, host phytochemical metabolism and insecticide metabolism provides a possible target for control at all stages of growth; naturally occurring synergists that inactivate these enzymes should render the NOW more sensitive not only to insecticides, allowing the use of lower dosages, but also to host phytochemicals and mycotoxins.

Certain constituents of herbs, spices and other plants are known to inhibit growth of aflatoxin-releasing fungi (Rusul and Marth, 1988). Among them, essential oils in particular suppress growth and aflatoxin formation by *Aspergillus* spp. (Belzile et al., 2000; Razzaghi-Abyaneh et al., 2008; Singh et al., 2009; Shukla et al., 2009; Nogueira et al., 2010). Myristicin (MYR, Fig. 1), a compound found in essential oils of several plant species, inhibits the biosynthesis of aflatoxin G1 (AFG1) in *Aspergillus parasiticus* (Razzaghi-Abyaneh et al., 2007). Other important plant-derived aflatoxin-inhibitory compounds summarized by Razzaghi-Abyaneh et al. (2010) include phenolics (caffeic acid, 3,4-digalloyl quinic acid, chlorogenic acid) from *Pistacia vera* (pistachio), flavonoids (khellin, visnagin) and coumarins (xanthotoxin, bergapten, and psoralen) from seeds of *Ammi visnaga* (toothpick weed), one phenylpropanoid (dillapiol) from *Anethum graveolens* (dill) and two phenylpropanoids (apiol, myristicin) from *Petroselinum crispum* (parsley), phenolics
(ferulic acid, vanillic acid, p-coumaric acid) and flavonoids (eriodyctyol, 5,7-dihydroxycromone) from Arachis hypogaea (peanut), phenolics (thymol, carvacrol) from Satureja hortensis (summer savory), alkaloids from seeds of Piper longum (long pepper), phenolics (vanillic acid, catechin, 4-hydroxybenzoic acid, protocatechuic acid) from kernels of Prunus dulcis (almond) and spiroether from Matricaria recutita (German chamomile). A new potentially safe and sustainable approach for managing NOW in almond orchards may be to use natural essential oil synergists to simultaneously suppress caterpillar growth and mycotoxin production in almond orchards. Identifying synergistic activity of essential oil constituents “generally recognized as safe” (GRAS) (http://www.fda.gov/Food) could lead to novel approaches to sustainably manage NOW in both conventional and organic almond production.

In this study, we set out to ascertain the ability of essential oil constituents to synergize the toxicity of mycotoxins, hostplant phytochemicals and insecticides and subsequently reduce survival of NOW. Some naturally occurring essential oil constituents, such as myristicin, are known synergists for certain synthetic insecticides (Lichtenstein and Casida, 1963) and phytochemicals (Berenbaum and Neal, 1985; Neal, 1989; Neal and Berenbaum, 1989). Piperonyl butoxide (PBO), a well-known synthetic inhibitor of P450s and MYR were tested for their synergistic activities toward xanthotoxin (XAN) (a constituent of figs) (Murray et al., 1982), α-cypermethrin (CPE) and τ-fluvalinate (FLV) (two pyrethroid insecticides), methoxyfenozide (MFZ) (an insecticidal ecdysteroid agonist), and AFB1.

2. Materials and Methods

2.1 Chemicals

PBO was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). AFB1, MYR (78% purity), CPE, FLV, and XAN were purchased from Sigma Chemicals (St. Louis, MO). Methoxyfenozide was obtained from Helena Chemical Company (Memphis, TN). All of these chemicals were dissolved in methanol (MeOH) except AFB1, which was dissolved in analytical grade dimethyl sulfoxide (DMSO), and stored at -20°C. DMSO and MeOH were obtained from Fisher Scientific (Pittsburgh,
2.2 Insects

A laboratory colony of *A. transitella* originating from an almond orchard in Fresno, California that has been maintained for the past 25 years by the Commodity Protection and Quality Unit, USDA/ARS (Parlier, CA, USA), was kept in an insectary at 28 ± 4°C with 16:8 L:D cycle. Larvae were mass-reared until pupation in 500 ml plastic containers containing 200 g of a wheat bran diet (Finney and Brinkman, 1967). For bioassays, the newly hatched insects were transferred to artificial diets in a 1 oz (30 ml) plastic cup. Adults were transferred to a clean 32 oz (900 ml) cup with a piece of tissue paper placed inside the cup and a piece of tissue paper covering the top.

2.3 Insecticide toxicity bioassays

The toxicity of the three insecticides, CPE, MFZ and FLV, was determined by administering them in varying concentrations of insecticides in artificial diets to newly hatched larvae. A series of doses of insecticides (CPE, 10, 20, 50, 100, 300 and 500 ng/g; FLV, 0.2, 0.5, 1, 3 and 5 μg/g; MFZ, 20, 50, 100, 300 and 500 ng/g) were dissolved in MeOH and added to the artificial diet; for controls, an equal amount of MeOH was added to the artificial diet. For each bioassay series, twenty newly hatched larvae (collected between 0-12 hr after hatching) were raised on each single dose of insecticide and their mortality was recorded at 24 hr and 48 hr. The larvae that did not move when touched with a soft brush were considered as dead. The median lethal concentration (LC50) at 24 hr and 48 hr was calculated using the Probit Analysis function in SPSS v17 software (SPSS Inc., Chicago, IL).

2.4 Synergist assays

For the synergist bioassays, the three insecticides (CPE, FLV, MFZ), XAN and AFB1 were supplemented with MYR and PBO to determine the synergistic effects on NOW larvae. The final concentrations of the synergists MYR (25 μg/g) and PBO (200 μg/g) were determined to be nontoxic for first instar larvae. The concentrations selected for the insecticides and XAN caused 30-70% mortality to the newly hatched larvae after 36 hr. The dose of AFB1 (50 μg/g) was sublethal to larvae.
For the insecticide assays, the diets containing 25 μg/g CPE, 2 μg/g FLV or 50 ng/g MFZ in the presence or absence of MYR (25 μg/g) or PBO (200 μg/g) were fed to the larvae. Because high concentrations of XAN stock dissolved in MeOH crystallized immediately after being added to the artificial diets and made homogeneous incorporation difficult, XAN powder was mixed with the sucrose to be added to the diet, ground with a mortar and pestle until it was homogeneous, and then added directly to the diets to a final concentration of 2 mg/g XAN. The controls for the XAN assays were plain diet, plain diet with MeOH, and plain diet supplemented with 25 μg/g MYR or 200 μg/g PBO. Mortality was recorded daily until more than 80% of the treated insects were dead. For AFB1 assays, caterpillars were placed on diets containing 0.5% DMSO (control), 25 μg/g MYR, 200 μg/g PBO, 50 μg/g AFB1, 50 μg/g AFB1 + 200 μg/g PBO or 50 μg/g AFB1 + 25 μg/g MYR. Larval mortality was recorded every day and the larvae were weighed after 24 days. Each bioassay included 20 first instar larvae subjected to a single concentration of these chemicals and all bioassays were repeated at least three times.

2.5 Statistical analysis

The mean mortalities and errors at the different time intervals were calculated and differences between the chemical alone and the chemical administered with PBO or MYR were determined by the post hoc LSD test that was part of the one-way analysis of variance (one-way ANOVA) module in the SPSS v17 software. Differences among mean larval weights were also compared in the AFB1 assays using the post hoc LSD test following one-way analysis of variance (ANOVA).

3 Results

3.1 Comparison of the toxicity of the insecticides

The insecticides examined in this study exhibited differential toxicity to NOW. Among pyrethroids, CPE is 26-fold more toxic at 24 hr and 56-fold more toxic at 48 hr than FLV. The ecdysteroid agonist insecticide, MFZ, began to cause mortality at 36 hr when the larvae started molting. The toxicity at 48 hr was lower than CPE (0.17-fold) and higher than FLV (9.6-fold) (Table 2).

3.2 Synergistic effects
The P450 inhibitors PBO and MYR were applied to test synergistic effects on the toxicity of the insecticides, phytochemicals and aflatoxins to the newly hatched NOW larvae. Both PBO and MYR synergized CPE; PBO significantly increased larval mortality starting from 12 hr and MYR significantly increased mortality starting from 4 days. For the other pyrethroid insecticide, FLV, only PBO continuously showed significant synergistic effects over time (Fig. 1B). MFZ, a substituted dibenzoylhydrazine, affects the molting process and causes toxicity to the insects during larval development (Carlson et al., 2001). Following the addition of 25 μg/g MYR and 200 μg/g PBO to diets containing 50 ng/g MFZ, there was no significant increase in mortality (Fig. 1C).

Following range finding assays, a 2 mg/g dose of XAN was selected because it caused at least 20% mortality of first instar larvae after 2 days. To test synergism of XAN with PBO and MYR, diets containing 2 mg/g XAN were supplemented with 25 μg/g MYR or 200 μg/g PBO. The results showed that PBO significantly increased mortality of XAN after 3 days (Fig. 1D). The diets with XAN alone caused 16% and 30% mortality, respectively, after 3 and 4 days, in contrast with 32% and 61% for XAN plus PBO (P =0.02 for the third day, P =0.0001 for the fourth day). MYR might slightly synergize XAN because mortality caused by XAN plus MYR was consistently higher over time, but a significant synergistic effect was recorded only on the seventh day, when the mortality of the larvae treated with XAN plus MYR (83%) was higher than that of the larvae treated with XAN (65%) (p =0.01).

Bioassays in which effects on larval mortality are recorded as an indicator of toxicity are not as robust in testing the synergistic effects on AFB1 toxicity to NOW because these larvae can tolerate extremely high levels of aflatoxins (Niu et al., 2009). Even a massive dose of AFB1 (over 50 μg/g) did not kill the insects immediately and many larvae survived long after this initial exposure to AFB1. In addition to measuring mortality, larval weights were recorded as a supplemental indicator of AFB1 toxicity, in treatments where either 25 μg/g MYR or 200 μg/g PBO along with 50 μg/g AFB1 were added to the diet, and daily mortality and larval
weights after 24 days were compared. The results (Table 3) indicate that neither PBO+AFB1 nor MYR+AFB1 caused any significant increase in mortality or significant decrease in larval weight.

4. Discussion

A combination of chemical sprays, biological controls and cultural control is used to minimize NOW damage in orchards in California. Pyrethroid insecticides, such as permethrin, bifenthrin, beta cyfluthrin and lambda cyhalothrin have been widely used in controlling infestations in pistachios, with no insecticide resistance yet reported for NOW. In this study we demonstrate that CPE can also effectively kill NOW larvae, and that FLV, which is frequently used to control aphids and mites in beehives (Johnson et al., 2006) and is not generally as toxic as CPE, can also effectively kill NOW larvae. MFZ, frequently used in almonds because it is an insect growth regulator that inhibits the larval molting process in target lepidopteran pests and is not harmful to beneficial insects and mites (Carlson et al., 2001), can effectively cause mortality in NOW larvae, although mortality occurs at a slower rate than CPE and FLV.

Pyrethroid insecticides are known to be metabolized by detoxificative P450s and esterases in several insect species (Ishaaya 1993; Pilling et al., 1995; Johnson et al., 2006). In our studies, PBO, a well-known synergist that inhibits many (but not all) insect P450s, significantly synergized the toxicity of both pyrethroids (CPE and FLV) to NOW larvae. MYR, a natural phytochemical previously demonstrated to be capable of synergizing P450-mediated detoxification, increased the toxicity of only a single insecticide tested, CPE. These data reaffirm that P450s are involved in metabolism of the pyrethroid insecticides by NOW and that inhibition of P450s can be a management strategy to increase the efficacy of the pyrethroid insecticides to control NOW. Because MYR also can inhibit growth of A. flavas and decrease formation of aflatoxins (Razzaghi-Abyaneh et al., 2007), it would appear to be an exceptional candidate for NOW control. It is relatively non-toxic to humans, it synergizes an insecticide (CPE), and it can help to reduce aflatoxin contamination in orchards.
Mosallanejad and Smagghe (2009) recently reported that PBO significantly synergized MFZ toxicity to the resistant strain of the cotton leafworm *Spodoptera littoralis*. In this particular study, the P450 activity in the resistant strain was increased 2.1-fold compared with the susceptible strain and it may be that the reported synergistic effects in the resistant strain were caused by inhibition of MFZ oxidation. Differences between *S. littoralis* and NOW larvae may exist if P450s are not the main detoxification system for MFZ in NOW or if the specific P450s mediating this process are resistant to PBO inhibition. Future research should examine the inhibitors of other detoxification enzymes, such as glutathione S-transferases or hydrolases, to determine if these pathways are involved in MFZ detoxification.

When AFB1 and XAN were evaluated, neither the naturally occurring essential oil constituent MYR nor the synthetic compound PBO synergized the toxicity of AFB1. This finding suggests that NOW may rely mainly on detoxification systems other than P450s to metabolize AFB1. Alternatively, this absence of synergism may be due to the fact that the NOW may possess excess metabolic capacity for detoxifying AFB1, which is frequently encountered in the fungus-contaminated hosts utilized by this lepidopteran. In contrast, both PBO and MYR synergized XAN toxicity. Although the mechanisms by which the NOW tolerates XAN and other furanocoumarins are as yet unknown, detoxification by P450s is a distinct possibility because these compounds are metabolized by P450s in three different families of lepidopterans (Oecophoridae, Noctuidae, Papilionidae). CYP6AB11 from NOW, expressed heterologously in a baculovirus system, is capable *in vitro* of metabolizing imperatorin, a furanocoumarin found in some species in the Rutaceae, a family containing some reported hosts of this species (Niu 2010). MYR is also known to synergize furanocoumarin toxicity in several other lepidopterans species (Berenbaum and Neal, 1985; Neal, 1989; Neal and Berenbaum, 1989; Yu and Hsu, 1993; Mao et al., 2008). In view of these findings, and in view of the limited availability of environmentally safe synthetic insecticides for sustainable management of NOW in almond organic orchards, MYR should be explored further.
as a field treatment to reduce survival of this pest species and aflatoxin contamination in orchard situations.

References


Table 1. Structures of the chemicals used in the bioassays.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>piperonyl butoxide</td>
<td><img src="image1" alt="Structure" /></td>
</tr>
<tr>
<td>myristicin</td>
<td><img src="image2" alt="Structure" /></td>
</tr>
<tr>
<td>α-cypermethrin</td>
<td><img src="image3" alt="Structure" /></td>
</tr>
<tr>
<td>τ-fluvalinate</td>
<td><img src="image4" alt="Structure" /></td>
</tr>
<tr>
<td>methoxyfenozide</td>
<td><img src="image5" alt="Structure" /></td>
</tr>
<tr>
<td>xanthotoxin</td>
<td><img src="image6" alt="Structure" /></td>
</tr>
<tr>
<td>aflatoxin B1</td>
<td><img src="image7" alt="Structure" /></td>
</tr>
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</table>
Table 2. LC$_{50}$ of insecticides for first instar *Amyelois transitella*. Mortality of first instars treated with varying concentrations of insecticides was recorded after 24 and 48 hours. All bioassays were repeated at least three times and the LC$_{50}$ (the concentration of the insecticide in the diets that causes 50% mortality at the specified time) was calculated with the Probit analysis function from SPSS v17 software (SPSS Inc., Chicago, IL).

<table>
<thead>
<tr>
<th>Insecticides</th>
<th>24h LC$_{50}$ (95% Confidence Limits) µg/g</th>
<th>48h LC$_{50}$ (95% Confidence Limits) µg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>τ-fluvalinate</td>
<td>1.9 (1.2-3.0)</td>
<td>0.73 (0.45-1.2)</td>
</tr>
<tr>
<td>α-cypermethrin</td>
<td>0.072 (0.045-0.11)</td>
<td>0.013 (0.007-0.023)</td>
</tr>
<tr>
<td>methoxyfenozide</td>
<td>-</td>
<td>0.076 (0.043-0.13)</td>
</tr>
</tbody>
</table>
Table 3. Bioassays of potential synergists of aflatoxin B1 toxicity to first instar *Amyelois transitella*. Effects of MYR and PBO on the toxicity of AFB1 were quantified by measuring larval weight after 24 days. First instars received plain diet containing 0.5% DMSO, 50 µg/g AFB1, 200 µg/g PBO, 25 µg/g MYR, 50 µg/g AFB1 plus 200 µg/g PBO or 25 µg/g MYR. Mean survivorship or larval weight was compared between the treatment of 50 µg/g AFB1 and that of 25ng/g MYR+AFB1 or 200ng/g PBO+AFB1 with LSD tests of one-way analysis of variance (ANOVA) and no significant difference was found.

<table>
<thead>
<tr>
<th>Survival (%)</th>
<th>Larval weight (mg) on day 24</th>
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<tr>
<td><strong>plain diet</strong></td>
<td>89±10 91±16</td>
</tr>
<tr>
<td>0.25% DMSO</td>
<td>90±7 87±22</td>
</tr>
<tr>
<td>50 µg/g AFB1</td>
<td>88±13 17±8</td>
</tr>
<tr>
<td>25ng/g MYR+AFB1</td>
<td>83±7 19±12</td>
</tr>
<tr>
<td>200ng/g PBO+AFB1</td>
<td>74±15 17±13</td>
</tr>
<tr>
<td>25ng/g MYR</td>
<td>83±10 89±20</td>
</tr>
<tr>
<td>200ng/g PBO</td>
<td>100 91±19</td>
</tr>
<tr>
<td>Time (h)</td>
<td>0h</td>
</tr>
<tr>
<td>---------</td>
<td>----</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>0</td>
</tr>
<tr>
<td>plain diet</td>
<td></td>
</tr>
<tr>
<td>MeOH</td>
<td></td>
</tr>
<tr>
<td>PBO</td>
<td></td>
</tr>
<tr>
<td>MYR</td>
<td></td>
</tr>
<tr>
<td>25ng/g CPE</td>
<td></td>
</tr>
<tr>
<td>PBO+CPE</td>
<td></td>
</tr>
<tr>
<td>MYR+CPE</td>
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</table>

Fig. 1 Synergistic effects of piperonyl butoxide (PBO) and myristicin (MYR) on insecticide and xanthotoxin (XAN) toxicity to navel orangeworm. Fig. 1A Effects of PBO and MYR on the mortality of newly hatched larvae exposed to cypermethrin (CPE) (Fig. 1A), fluvalinate (FLV) (Fig. 1B), methoxyfenozide (MFZ) (Fig. 1C) and XAN (Fig. 1D) were tested. Larvae were fed with artificial diets containing 25 ng/g CPE, 2 μg/g FLV, 50 ng/g MFZ or 2 mg/g XAN supplemented with or without 25 μg/g MYR or 200 μg/g PBO. Plain diet and diets containing the synergists alone or an equal amount of MeOH were used as controls. All of the bioassays were repeated at least three times. The mean mortality was compared between treatments with LSD tests of one-way analysis of variance (ANOVA).
Fig. 1B. Effects of PBO and MYR on the mortality of newly hatched larvae exposed to fluvalinate (FLV).
Fig. 1C. Effects of PBO and MYR on the mortality of newly hatched larvae exposed to methoxyfenozide (MFZ).
Fig. 1D. Effects of PBO and MYR on the mortality of newly hatched larvae exposed to xanthotoxin (XAN).
Vita

Guodong Niu was born in Changzhi, China on Aug 13, 1977, the son of Haijiang Niu and Huifang Zhao. He earned a B.A. in Environmental Science from Shanxi University in Taiyuan, China. He was awarded his M. S. in Microbiology in 2000 in Wuhan Institute of Virology, Chinese Academy of Sciences, after completion of a thesis project on the study of molecular evolution and functional analysis of ubiquitin of Spodoptera exigua Multi Nucleocapsid Nucleopolyhedrovirus. His Ph.D. research focus is on insects resistant against fungal toxins and the role of the cytochrome P450 monooxygenases involved in detoxification and bioactivation of mycotoxins. Following completion of his Ph.D., Guodong will pursue post-doctoral studies in insect biochemistry and molecular biology, chemical ecology of plant-insect interactions.
Publications


