

Toxicity of Aflatoxin B1 to *Helicoverpa zea* and Bioactivation by Cytochrome P450 Monooxygenases

Ren Sen Zeng · Guodong Niu · Zhimou Wen ·
Mary A. Schuler · May R. Berenbaum

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Abstract Infestation of corn (*Zea mays*) by corn earworm (*Helicoverpa zea*) predisposes the plant to infection by *Aspergillus* fungi and concomitant contamination with the carcinogenic mycotoxin aflatoxin B1 (AFB1). Although effects of ingesting AFB1 are well documented in livestock and humans, the effects on insects that naturally encounter this mycotoxin are not as well defined. Toxicity of AFB1 to different stages of *H. zea* (first, third, and fifth instars) was evaluated with artificial diets containing varying concentrations. Although not acutely toxic at low concentrations (1–20 ng/g), AFB1 had significant chronic effects, including protracted development, increased mortality, decreased pupation rate, and reduced pupal weight. Sensitivity varied with developmental stage; whereas intermediate concentrations (200 ng/g) caused complete mortality in first instars, this same concentration had no detectable adverse effects on larvae encountering AFB1 in fifth instar. Fifth instars consuming AFB1 at higher concentrations (1 µg/g), however, displayed morphological deformities at pupation. That cytochrome P450 monooxygenases (P450s) are involved in the bioactivation of aflatoxin in this species is evidenced by the effects of piperonyl butoxide (PBO), a known P450 inhibitor, on toxicity; whereas no fourth instars pupated in the presence of 1 µg/g AFB1 in the diet, the presence of 0.1% PBO increased the pupation rate to 71.7%. Pupation rates of both fourth and fifth instars on diets containing 1 µg/g AFB1 also increased significantly in the presence of PBO. Effects of phenobarbital, a P450 inducer, on

R. S. Zeng (✉) · G. Niu · M. R. Berenbaum
Department of Entomology,
University of Illinois, Urbana, IL 61801, USA
e-mail: rszeng@life.uiuc.edu

R. S. Zeng
Department of Ecology,
South China Agricultural University, Guangzhou 510642, China

Z. Wen · M. A. Schuler
Department of Cell and Development Biology,
University of Illinois, Urbana, IL 61801, USA

AFB1 toxicity were less dramatic than those of PBO. Collectively, these findings indicate that, as in many other vertebrates and invertebrates, toxicity of AFB1 to *H. zea* results from P450-mediated metabolic bioactivation.

Keywords Aflatoxin B1 · Bioactivation · Cytochrome P450 monooxygenase · *Helicoverpa zea* · Mycotoxin · Piperonyl butoxide

Abbreviations

AFB1	aflatoxin B1
P450	cytochrome P450 monooxygenase
AFBO	AFB1-8,9-epoxide
AFM1	aflatoxin M1
AFL	aflatoxicol
AFQ1	aflatoxin Q1
AFB2a	aflatoxin B2a
AFG1	aflatoxin G1
PBO	piperonyl butoxide
PB	phenobarbital

Introduction

Helicoverpa zea, the corn earworm, is a major pest of corn and a number of other crops throughout the USA; current estimates indicate that this species alone is responsible for losses exceeding 10% in field corn and 50% in sweet corn (Wiseman, 1999). Losses inflicted by *H. zea* infestation can be exacerbated by fungal infections that may accompany *H. zea* damage (Dowd and White, 2002). Among the most devastating are the ear molds that produce aflatoxins, including some of the most active natural carcinogens known (Lillehoj, 1992; Dowd, 1998). Because the maximum tolerance levels for aflatoxins are in the 0- to 20-ppb range for corn grown for human consumption, aflatoxin contamination in the USA is responsible for millions of dollars of crop losses every year [U.S. Department of Agriculture (USDA), 1999].

Aflatoxins are coumarin derivatives that can be classified as either dihydrofurocyclopentenones or dihydrofurolactones. These compounds owe their toxicity to their ability to form irreversible adducts to nucleic acids with the concomitant inhibition of DNA replication and DNA-dependent transcription (Murray et al., 1982). Chronic effects of exposure include decreases in food consumption in pigs, poultry, cattle, and a variety of livestock as well as decreases in milk yields in cattle. Acute and chronic exposures result in a variety of clinical symptoms in humans, including pulmonary edema, abdominal pain, vomiting, convulsions, and, in some cases, coma and death (Newberne and Butler, 1969).

One means of reducing the risk of aflatoxin contamination of corn is to reduce the likelihood of insect damage, which predisposes the plant to fungal infection. Reduction of insect damage through insecticide application and the use of insect-resistant germ plasm (e.g., tight-husked varieties) has had limited success owing to the evolution of resistance to both forms of control (Dowd et al., 1997). Transgenic Bt corn has long been touted as another means of reducing crop damage, but it is unclear how effective it will be (Windham et al., 1999), given that *H. zea* displays a wide range of sensitivity to Bt (Stone and Sims, 1993), and that any level of insect

infestation can facilitate mold establishment leading to mycotoxin contamination of corn (Dowd, 2001).

Although aflatoxins are toxic to a broad range of arthropods (Al-Adil et al., 1972; Wright et al., 1982; Chinnici and Bettinger, 1984; Lillehoj, 1992; Sadek et al., 1996), comparatively little is known about metabolic transformations of aflatoxin in insects that regularly encounter them. Among the enzyme systems responsible for metabolizing these compounds in many vertebrate and invertebrate taxa are the cytochrome P450 monooxygenases (P450s; <http://drnelson.utmem.edu>; <http://www.biobase.dk/P450/>). Whereas in some species P450s metabolize aflatoxin B1 (AFB1) to hydroxylated metabolites, including aflatoxin M1 (AFM1), aflatoxin G1 (AFG1), and aflatoxin Q1 (AFQ1), in other species, P450s are responsible for bioactivation of these compounds, catalyzing the epoxidation of the terminal furan ring of AFB1 to yield AFB1-8,9-epoxide (AFBO), a highly genotoxic metabolite (Murray et al., 1982). AFBO owes its toxicity to its ability to bind to DNA to form the predominant 8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB1 adduct (Iyer et al., 1994). Hydroxylated metabolites have lower genotoxic or toxic activities than AFB1 in most organisms (Eaton et al., 1988; Ramsdell and Eaton, 1990). The Hikone-R strain of *Drosophila melanogaster*, which is resistant to DDT and other insecticides, produces aflatoxicol (AFL) as a principal metabolite, along with AFM1 and aflatoxin B2a (AFB2a) (Foerster and Wurgler, 1984). Saner et al. (1996) demonstrated that bioactivation of AFB1 was catalyzed by CYP6A2. Lee and Campbell (2000) compared metabolism of AFB1 in larvae of *Amyelois transitella* (navel orangeworm), which are frequently exposed to aflatoxin-contaminated tissue, and *Cydia pomonella* (codling moth), which are rarely exposed to aflatoxins. These authors found that AFL is the major metabolite produced *in vitro* by navel orangeworms; in contrast, codling moths collected in the field produced only trace amounts of AFL, and a laboratory strain produced no detectable metabolites. Neither species produced AFBO as a metabolite, in contrast to vertebrates, which produce this highly mutagenic biotransformation product quite readily. Lee and Campbell (2000) have suggested that the absence of this particular bioactivation is indicative of coevolution between kernel-feeding insects and these toxin-producing fungi.

Although *H. zea* is likely to encounter aflatoxins in corn, as well as in several other host plants (including soybeans *Glycine max*), the degree to which this species has coevolved with mycotoxin-producing *Aspergillus* species is unknown. In this study, we examined the toxicological impact of AFB1 ingestion throughout the larval developmental period and tested the effects of a known inhibitor of cytochrome P450 metabolism, piperonyl butoxide (PBO), as well as a known P450 inducer, phenobarbital (PB; Brun et al., 1996; Maitra et al., 1996; Dunkov et al., 1997; Waxman 1999; Stevens et al., 2000; Li et al., 2002a; Brown et al., 2003), to determine the contributions of these enzymes to aflatoxin toxicity in this species.

Methods and Materials

Chemicals

Aflatoxin B1 and PB were obtained from Sigma (St. Louis, MO, USA). Analytical grade dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific, and PBO was from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

Insects

Helicoverpa zea were individually reared in 30-ml creamer containers on a semisynthetic diet containing wheat germ (Waldbauer et al., 1984) and maintained in an insectary kept at 23–26°C with a photoperiod of 16-hr light/8-hr dark. Bioassays were conducted under the same environmental conditions. At each developmental stage tested, caterpillars were collected within 12 hr of molting to insure age comparability.

Bioassays

Stock solutions of AFB1 were prepared in DMSO at concentrations of 1 ng/μl, 20 ng/μl, 200 ng/μl, 1 μg/μl, and 20 μg/μl; 100 μl of each stock solution were added to 100 g of the semisynthetic diet to obtain final concentrations of 1 ng/g, 20 ng/g, 200 ng/g, 1 μg/g, and 20 μg/g AFB1. Control larvae were fed with the semisynthetic diet containing 100 μl DMSO per 100 g; pilot experiments determined that concentrations of DMSO at levels as high as 0.5% of the diet do not affect development or survivorship (data not shown).

To determine the toxicity of aflatoxin to *H. zea*, artificial diets containing AFB1 at four concentrations were administered in separate bioassays to larvae at three developmental stages—first, third, and fifth instars; 20 larvae were tested in each bioassay. Because pilot studies established that early instars display higher sensitivity to AFB1 (data not shown), first instars were exposed to diets containing AFB1 at concentrations of 1 ng/g, 20 ng/g, 200 ng/g, and 1 μg/g, and third and fifth instars were exposed to diets containing AFB1 at concentrations of 20 ng/g, 200 ng/g, 1 μg/g, and 20 μg/g. The proportions of larvae at each stage of development and the number of dead larvae were recorded daily through pupation, and pupal weights were measured within 48 hr of pupation. Each set of bioassays was replicated three times.

To elucidate the effects of cytochrome P450-mediated metabolism on aflatoxin toxicity, a series of bioassays was conducted whereby AFB1 toxicity was assessed in the presence of an inducer (PB) or an inhibitor (PBO). Stock solutions of PB were prepared in DMSO at a concentration of 0.25 mg/μl PB, and 400 μl of this stock were added to 100 g of the semisynthetic diet to obtain a final concentration of 0.1% PB in the diet. An aliquot of 105 μl commercial PBO (90% purity, 1.06 density) was added directly to 100 g of the diet for a final concentration of 0.1% PBO. Newly molted fourth or fifth instars were assayed separately, with 20 larvae per treatment. Caterpillars were placed on diets containing either 0.1% DMSO (control), 0.1% PBO, 0.1% PB, 1 μg/g AFB1, 1 μg/g AFB1 + 0.1% PBO, or 1 μg/g AFB1 + 0.1% PB and were reared and evaluated as described.

Statistical Analyses

All data were evaluated by one-way analysis of variance with treatment differences among means tested at $P = 0.05$ with Duncan's multiple range test. All data for larval development, pupation rate, and mortality were means from three replicates, with 20 caterpillars/treatment. Because significant differences were found among replicates in pupal weights, this parameter was analyzed independently for each replicate, and representative values from one replicate are reported.

Safety

Aflatoxin B1 is a highly toxic mycotoxin that should be handled with care. Solid AFB1 for calibration should be handled in a biohood, using a nose and mouth mask. Any material and equipment with possible contamination should be handled carefully and with surgical gloves.

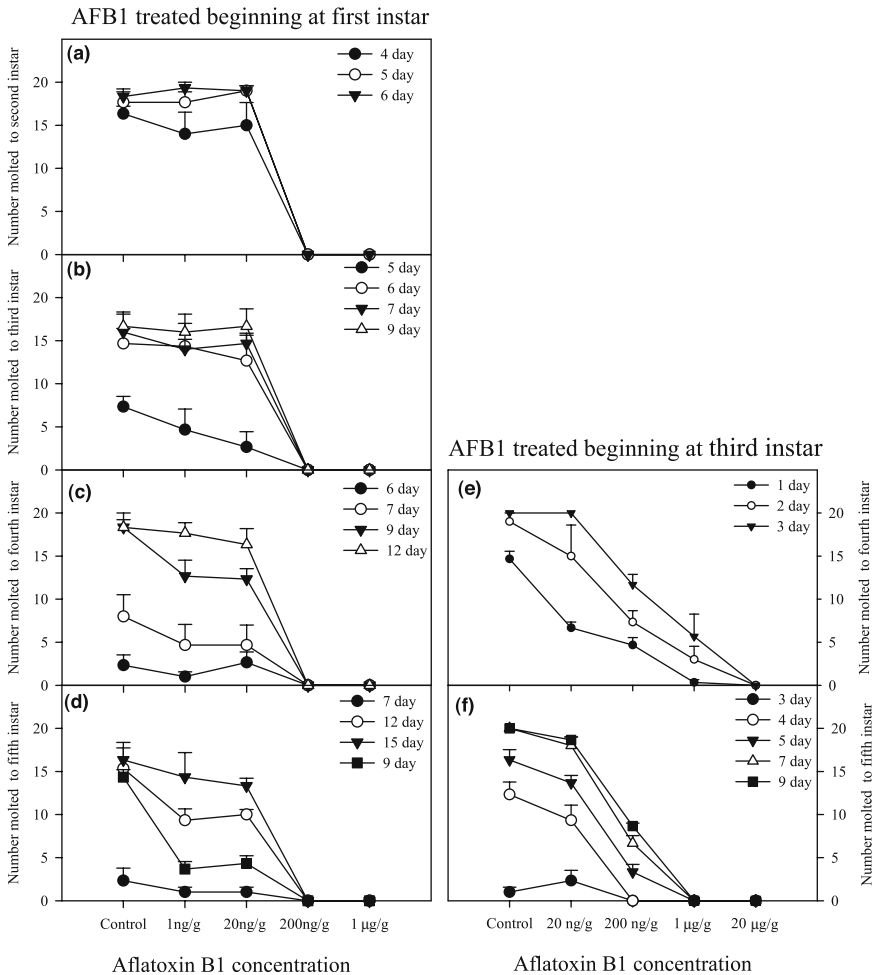


Fig. 1 Development of *H. zea* after exposure to different concentrations of aflatoxin B1 in diet beginning at first instar (a, b, c, d) and third instar (e, f). (a) Number of larvae that have molted to second instar after 4, 5 and 6 days of treatments, (b) number of larvae that have molted to third instar after 5, 6, 7 and 9 days of treatments, (c) number of larvae that have molted to fourth instar after 6, 7, 9 and 12 days of treatments, (d) number of larvae that have molted to fifth instar after 7, 9, 12 and 15 days of treatments, (e) number of larvae that have molted to fourth instar after 1, 2 and 3 days of treatments, (f) number of larvae that have molted to fifth instar after 3, 4, 5, 7 and 9 days of treatments. Each treatment contained 20 caterpillars and each experiment was repeated three times. Values are means + standard errors. Points on each line represent effects of different concentrations of AFB1 on a given day after AFB1 exposure

Results

Toxicity to First Instars

AFB1 at high concentrations strongly inhibited larval growth and development. At a concentration of 200 ng/g, larval mortality was 55% after 9 d ($P = 0.02$) and 100% after 15 d ($P < 0.001$; Figs. 1a–d and 2a). Most larvae died before molting to second instar, and only about 10% of larvae survived to third instar after 12 d. AFB1 at a concentration of 1 $\mu\text{g/g}$ completely inhibited larval development, and all larvae had died within 12 d without molting to second instar ($P < 0.001$; Fig. 2a).

AFB1 at the lower concentrations of 1 and 20 ng/g did not detectably delay the timing of the molt to second instar (Fig. 1a) but did delay developmental progress at all subsequent stages (Fig. 1b–d). Low concentrations of AFB1 also significantly reduced pupation rate ($P = 0.004$ at 1 ng/g and $P < 0.001$ for 20 ng/g) and pupal weight ($P = 0.001$ for 1 ng/g and $P < 0.001$ for 20 ng/g; Fig. 3a and b); after 15 d, mortality was 46.6% on diets containing 20 ng/g AFB1 compared with 18.3% on control diet ($P = 0.029$; Fig. 2a).

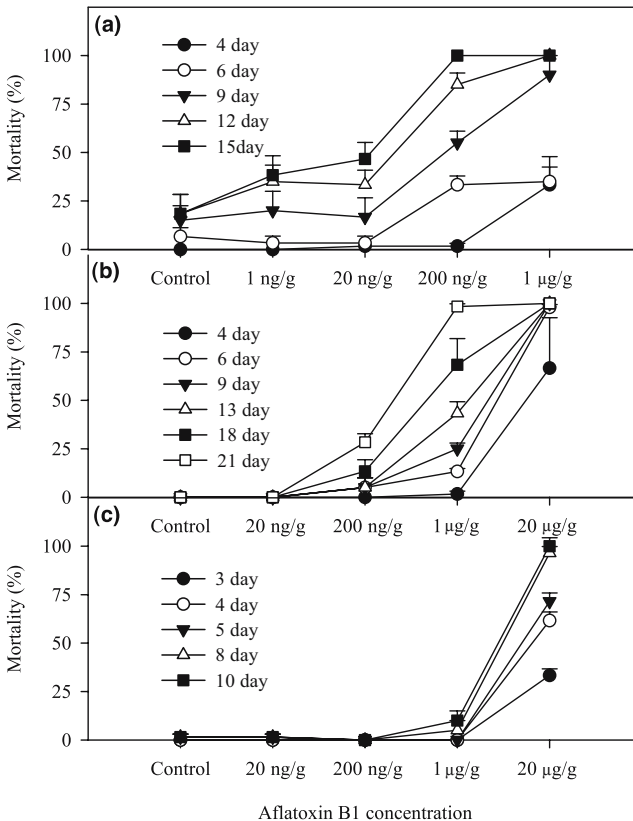


Fig. 2 Mortality of *H. zea* after exposure to different concentrations of aflatoxin B1 in diet beginning at first instar (a), third instar (b), and fifth instar (c). Values are means + standard errors from 20 caterpillars/treatment with three experimental replicates for each

Toxicity to Third Instars

Third instars display greater tolerance to AFB1 than do first instars. Administered to third instar larvae, the lowest concentration (20 ng/g) of AFB1 delayed development as well as pupation (Figs. 1e,f and 3c); pupal weight was also significantly reduced (Fig. 3d), although pupation rate and mortality were unaffected (Figs. 2b and 3c). The intermediate concentration of 200 ng/g AFB1 significantly delayed development (Figs. 1e,f and 3c) and reduced pupal weight to the same extent as did diets containing 20 ng/g AFB1 ($P = 0.411$; Fig. 3d). Mortality rates assessed after 21 d of treatment were 28.5% compared to no mortality for control larvae ($P < 0.001$; Fig. 2b) and were significantly lower than the mortality rate of 55% for first instars consuming AFB1 at this concentration ($P < 0.001$) for 9 d. Pupation rates assessed after 28 d of treatment were 66.7% compared to 100%

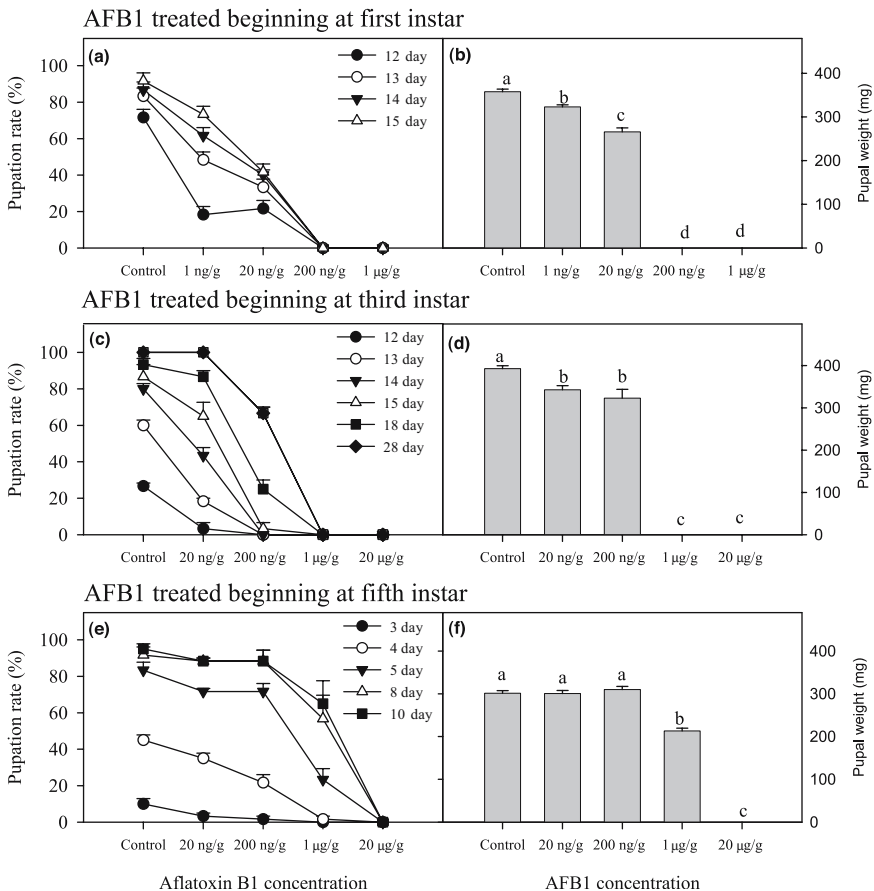


Fig. 3 Pupation of *H. zea* after exposure to different concentrations of aflatoxin B1 in diet beginning at first instar (a, b), third instar (c, d), and fifth instar (e, f). Values for pupation rate are means + standard errors from 20 caterpillars/treatment with three experimental replicates for each. Values for pupal weight are representative of one of the three series of replicates with the number of insects that have pupated for this replicate shown above each bar. Significant differences ($P < 0.05$) in pupal weights among treatments are indicated by different letters above the bars

for control larvae (Fig. 3c). Of the caterpillars that survived the growth delays associated with the treatment of third instars with 200 ng/g AFB₁, several reached pupal weights as high as those on control diets in all three replicate experiments.

Concentrations of 1 µg/g AFB₁ in the diet significantly interfered with development; only about 5% of larvae reached fourth instar, and of these, none developed into fifth instars (Figs. 1e, f and 3c). After 13 d, the larval mortality rate was 44.3%, and after 3 wk, no larvae remained alive (Fig. 2b). At the highest concentration (20 µg/g), no larval development occurred, and mortality was 100% after 6 d of treatment (Figs. 1e, f and 2b).

Toxicity to Fifth Instars

Fifth instar *H. zea* display substantially greater tolerance to AFB₁ than do earlier stages. Administered to fifth instars, the lowest concentrations (20 and 200 ng/g) of AFB₁ delayed pupation only after five or more days of treatment (Fig. 3e) without affecting pupation rate ($P = 0.479$ and 0.499), pupal weight ($P = 0.454$ and 0.245), or mortality ($P = 0.479$ and 0.499 ; Figs. 2c and 3e, f). At concentrations ≥ 1 µg/g AFB₁, however, fifth instar growth and development were affected. At a concentration of 20 µg/g, AFB₁ effected 100% mortality after 10 d (Fig. 2c). Whereas most larvae (83.3%) on control diets had pupated after 5 d, less than one third of larvae on diets containing 1 µg/g AFB₁ had pupated over that time period ($P = 0.011$). In addition, pupal weights on diets containing 1 µg/g AFB₁ were reduced by 29.3% relative to control diets ($P < 0.001$).

Morphological examinations indicated that malformations of pupae occurred when fifth instars consumed diets with 1 µg/g AFB₁ (Fig. 4); in these cases, the pupal molt is arrested so that only the anterior portion of the pupa, from the maxillary region to the anterior abdominal segments, protrudes dorsally from the thoracic and anterior abdominal areas of the persistent larval cadaver. These deformed pupae failed to produce adult moths.

PBO and PB Effects

To determine the role of P450s in the activation and/or detoxification of AFB₁ in *H. zea*, bioassays were conducted by administering AFB₁ alone, AFB₁ in combination with PBO, a P450 inhibitor, or AFB₁ in combination with PB, an inducer of some P450 monooxygenases. As shown in Fig. 5a and b, exposure of fourth instars to 0.1% PBO significantly improved the pupation rates ($P < 0.001$) and pupal weights



Fig. 4 Pupae of *H. zea* without (control) and with aflatoxin B₁ (1 µg/g) in diet beginning at fifth instar

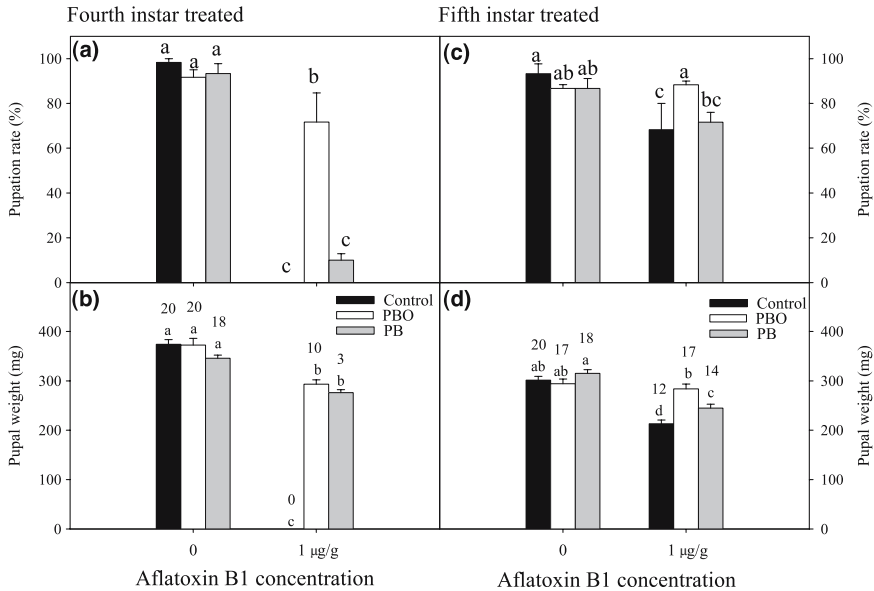


Fig. 5 Effects of piperonyl butoxide (PBO) and phenobarbital (PB) on pupation of *H. zea* exposed to aflatoxin B1 beginning at fourth instar (a, b) and at fifth instar (c, d). Values shown for pupation rate are means + standard error from three replicates with 20 caterpillars/treatment. Values for pupal weight are representative of one of the three series of replicates with the numbers of pupae weighed for this replicate shown above each bar. Significant differences ($P < 0.05$) among treatments are indicated by different letters above the bars

($P < 0.001$) of *H. zea* fed on diets containing 0.1% PBO and 1 µg/g AFB1 compared to *H. zea* on diets containing AFB1 alone. Whereas no fourth instars on diets containing 1 µg/g AFB1 succeeded in pupating, addition of PBO to the diet increased pupation rate of fourth instars to 71.7% ($P < 0.001$; Fig. 5a). In contrast, exposure of fourth instars to 0.1% PB resulted in a pupation rate of only 10% ($P = 0.256$). Pupal weights were not significantly changed by the addition of PB or PBO.

In contrast with fourth instars, which did not successfully pupate on diets containing 1 µg/g AFB1, over 68% of fifth instars on this diet succeeded in pupating. Inclusion of PBO in the diet increased pupation success to 88.3% ($P = 0.044$); at the same time, PBO increased pupal weights to an average of 283.7 mg, compared with an average of 213.0 mg on control diets ($P < 0.001$). Pupation success and pupal weights in the presence of AFB1 and PBO were not significantly different from pupation success and pupal weight of larvae on control diets (Figs. 5c and d; $P = 0.55$ for pupation rate and $P = 0.14$ for pupal weight). In contrast with PBO, addition of PB did not affect pupation rate, although addition of PB to diets containing AFB1 increased pupal weight significantly.

Discussion

Just as insects may possess biochemical mechanisms that confer resistance to insecticides and plant allelochemicals, they may also possess mechanisms for circumventing the toxicity of mycotoxins such as aflatoxins. In one study, resistance

levels among species varied with the likelihood of ecological exposure to these compounds (Lee and Campbell, 2000). Resistance levels may even vary intraspecifically; differences in AFB1 sensitivity were found in wild-type strains of *D. melanogaster* (Llwellyn and Chinnici, 1978; Chinnici and Melone, 1985). The Crimea strain was extremely sensitive, with no adults produced at concentrations of 0.4 mg/l AFB1, whereas eclosion rates exceeded 50% in three other resistant strains in the presence of the same concentrations of AFB1 (Llwellyn and Chinnici, 1978).

Although *H. zea* has a remarkable capacity for metabolizing a broad diversity of plant allelochemicals, as befits its polyphagous diet (Li et al., 2004), its ability to tolerate the mycotoxin aflatoxin is limited, despite its decidedly frequent ecological exposure. Although AFB1 was not acutely toxic to *H. zea* at any stage of development, even at the highest concentration tested (20 µg/g), chronic exposure led to developmental delays, reductions in pupation rates and pupal weights, and higher mortality. The chronic effects of AFB1 ingestion varied depending on the stage of larval exposure, with first instars the most susceptible to AFB1 and fifth instars the most resistant. Exposure of first instars to low levels (1 ng/g) of AFB1 significantly affected development time and pupation rate, whereas pupation rates of third and fifth instars were affected only at concentrations of 200 ng/g and 1 µg/g of AFB1. Exposure of first instars to concentrations of 20 ng/g, representing the maximum levels permitted in grain (USDA, 1998), resulted in prolonged development and reduced pupation rates. Similar developmental effects have been documented in other insects; growth of *D. melanogaster* on AFB1-containing media caused significant protraction in egg-to-adult developmental time (Lalor et al., 1976). In contrast, exposure of fifth instars to substantially higher levels (200 ng/g) of AFB1 did not affect pupation rate, pupal weight, or mortality. Developmental abnormalities in the form of pupal deformities, however, were observed at these higher concentrations, preventing eclosion. AFB1 has previously been reported to cause developmental aberrations, including decreased wing length, in adult *D. melanogaster* (Lalor et al., 1976).

With respect to the mode of action of AFB1, PBO, an effective inhibitor of P450s in many different phyla, significantly decreases the toxicity of AFB1 to *H. zea*, a result consistent with bioactivation of aflatoxin to more toxic derivatives by one or more P450s expressed in fourth and fifth instars. Bioactivation of this sort has been documented for AFB1 in human P450s, with CYP3A4 the predominant protein bioactivating AFB1 to its highly toxic AFB1 *exo*-8,9-epoxide derivative (Ueng et al., 1995); CYP2A6 contributes to some extent in the bioactivation process (Crespi et al., 1990; Yun et al., 1991). In the mouse (*Mus musculus*), bioactivation of AFB1 is mediated by CYP2A5, which is closely related to human CYP2A6 (Pelkonen et al., 1994).

Our results also demonstrate that PB, which is capable of inducing some mammalian (Waxman, 1999), insect (Brun et al., 1996; Maitra et al., 1996; Dunkov et al., 1997; Stevens et al., 2000; Li et al., 2002a), and plant (Batard et al., 1998; Persans et al., 2001) P450s, has only a marginal ability to induce the P450(s) capable of bioactivating aflatoxin in fourth instars and some ability to induce P450s, possibly responsible for AFB1 detoxification, that allow larvae to survive and gain weight on diets containing aflatoxin. In humans, CYP3A4 and, to a lesser extent, CYP1A2 contribute to the transformation of AFB1 to less toxic hydroxylated AFM1 and AFQ1 derivatives (Ueng et al., 1995; Guengerich et al., 1998). Among candidate

P450s in *H. zea* that may be involved in these bioactivation processes are CYP6B8, which is induced by a range of plant allelochemicals and signaling compounds as well as PB (Li et al., 2000, 2002a,b), CYP6B9 and CYP6B27, which are induced by plant allelochemicals and signaling molecules (Li et al., 2002a,b), and CYP321A1, which at present is known to be induced only by xanthotoxin (Sasabe et al., 2004). Of these, CYP6B8 and CYP321A1 are both capable of metabolizing a wide range of toxic allelochemicals and insecticides (Li et al., 2004; Sasabe et al., 2004).

Bioactivation of aflatoxins by P450s has been demonstrated as a necessary step in bringing about most of their toxic effects in humans and other animals (Eaton et al., 1994). P450s in corn earworm appear to be involved in both bioactivation and detoxification of AFB1. In that these P450s are induced in response to exposure to plant allelochemicals, bioactivated mycotoxins such as aflatoxins, which co-occur with plant allelochemicals that are detoxified by P450s, present a distinct toxicological challenge to *H. zea*. In view of the fact that insect damage predisposes plants to fungal infection, and that mycotoxins other than aflatoxins depend on P450-mediated metabolism for their toxic effects (e.g., ochratoxin A, Obrecht-Pflumio et al., 1999), bioactivation may account at least in part for negative effects of phytopathogens on herbivorous insects documented in many plant–insect interactions (Hatcher, 1995).

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