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FALL ARMYWORM SENSITIVITY TO FLAVONE: LIMITED ROLE OF CONSTITUTIVE AND INDUCED DETOXIFYING ENZYME ACTIVITY

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Abstract—We used inhibition and induction of detoxifying enzymes to determine whether these enzymes allow a generalist species (*Spodoptera frugiperda;* fall armyworms) to cope with ingestion of the flavonoid, flavone. Flavone induces polysubstrate monooxygenases (PSMO), general esterases (GE), and glutathione *S*-transferases (GST) in *S. frugiperda*, yet this species is affected deleteriously by low dietary concentrations of this allelochemical. First, in a series of experiments, larvae were fed artificial diets containing increasing concentrations of flavone, either alone or with known inhibitors of either PSMO, GE, or GST enzymes. In an additional treatment, flavone and inhibitors of all three enzyme systems were administered in diets simultaneously. PSMO and GE activities were reduced in vivo by their respective inhibitors, whereas that of GST was induced or unchanged. Significant synergism of flavone's growth-reducing activity occurred at the highest concentration tested (0.125% fresh mass, fm) when the PSMO inhibitor, piperonyl butoxide, or the GST inhibitor, diethyl maleate, was added to the diet, and at 0.08% fm flavone, when combined with the GE inhibitor, tri-tolyl phosphate. In many cases, however, the additive effect (i.e., reduction in growth owing to flavone alone + inhibitor alone) was greater than the synergistic effect, and no synergism occurred in the treatment with the three inhibitors combined. In the second approach, caterpillars were preexposed to a concentration of flavone (0.02% fm) that induced these enzymes ca. 1.5- to 2.5-fold, prior to switching larvae to a diet containing a higher (growth-reducing) flavone concentration (0.125% fm). The relative growth rates (RGR) of induced larvae were significantly greater (14%) than those of the uninduced larvae on the 0.125% fm flavone diet. Additionally, in two of the three experiments, relative consumption rate (RCR) was significantly greater (7–

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24%) in induced compared with uninduced larvae. The variable responses to inhibitor treatment and the relatively small benefit of enzyme induction suggest that these enzyme systems have minimal impact on the detoxification of flavone in *S. frugiperda*, even though this allelochemical induces enzyme activity and has been reported to be metabolized in vitro.

Key Words—Detoxification, *Spodoptera frugiperda*, Lepidoptera, Noctuidae, flavone, induction, polysubstrate monooxygenase, glutathione transferase, general esterase, synergism.

INTRODUCTION

Three systems of detoxifying enzymes [i.e., polysubstrate monooxygenases (PSMO), general esterases (GE), and glutathione *S*-transferases (GST)] are commonly regarded as the most important biochemical mechanisms for the metabolism of xenobiotics (Terriere, 1984; Brattsten, 1992) including allelochemicals (Yu, 1986) and pesticides (Hodgson et al., 1991). Xenobiotics may act as inducers by stimulating enzyme synthesis (Yu, 1986). Insects induced by dietary allelochemicals or host plants apparently increase metabolism of several synthetic pesticides, as demonstrated by their increased tolerance to these compounds (Hodgson et al., 1991). Insecticide-resistant strains of insects often have greater detoxifying enzyme activities (Soderlund and Bloomquist, 1990; Yu, 1991), and in at least one example, enzyme inducibility was greater than in a susceptible strain (Ottea and Plapp, 1984). In contrast, little is known about the role of detoxifying enzymes in the tolerance of allelochemicals by insects, although the in vivo metabolism of several allelochemicals has been demonstrated (Proksch et al., 1987; Nitao, 1989, 1990; Dowd, 1990; Harwood et al., 1990; Berge and Rosenthal, 1991; Smirle and Isman, 1992). Additionally, few studies have demonstrated increased allelochemical tolerance in insects attributable to induced enzyme activity (Brattsten et al., 1977; Kennedy, 1984; Gunderson et al., 1985).

Midgut preparations of allelochemical-induced caterpillars metabolize the inducing allelochemical more rapidly in vitro than do those of controls (Yu, 1986; Wadleigh and Yu, 1987, 1988a, b; Nitao, 1989; Harwood et al., 1990). However, to our knowledge only one published study demonstrates increased tolerance of an insect [*Helicoverpa zea* (Boddie)] to an allelochemical (2-tridecanone) following exposure to the same compound (Kennedy, 1984). Although monooxygenase enzyme activity was not measured in that study, induction probably occurred, as cytochrome P-450 content and GST activity were greater in larvae of another noctuid species *Heliothis virescens* (F.) after three days of feeding on diets containing 2-tridecanone compared with controls (Riskallah et al., 1986). For induced detoxifying enzyme activity to be an adaptive response mitigating the negative impact of xenobiotics, we suggest these enzymes should

protect insects from exposure to the same compounds that act as the inducing agents.

In this study we investigated whether there is a benefit of detoxifying enzyme activity to larvae of *Spodoptera frugiperda* (J.E. Smith) in terms of their ability to cope with the ingested allelochemical, flavone (Figure 1). Although this is a generalist species, reported from over 25 plant families (Tietz, 1972), it does not occur on plants known to contain flavone (e.g., *Primula* spp.). Therefore, we would not expect *S. frugiperda* to be specifically adapted to this allelochemical as monophagous species may be to the allelochemicals occurring in their food plants (e.g., Berenbaum et al., 1990). However, flavone represents the basic structure for several thousand flavonoids (Harborne, 1988), many of which occur in plants that comprise this species' host range (Tietz, 1972). Furthermore, this allelochemical is known to induce PSMO, GE, and GST enzyme activities 4.4-, 1.8-, and 3.3-fold, respectively (Yu, 1983, 1984; Yu and Hsu, 1985), in *S. frugiperda* and is metabolized in vitro by midgut preparations from this species (Yu, 1987). Thus, it is likely that *S. frugiperda* larvae have the capacity to detoxify flavone and other flavonoids. We used two approaches to investigate whether there is a benefit to constitutive and induced enzyme activities in terms of tolerating dietary flavone in *S. frugiperda* larvae.

First, caterpillars with inhibited detoxifying enzymes should perform more poorly than those with uninhibited enzymes when fed a performance-reducing dose of an allelochemical if the blocked enzymes normally detoxify the allelochemical. Thus, we used insecticide synergists in an attempt to block the major detoxifying enzyme systems and then measured the change in insect performance when fed a flavone-containing diet. Many reports describe in vitro assays consisting of these inhibitors and insect tissue preparations (e.g., Wadleigh and Yu, 1988a, b; Lee, 1991) but only a few inhibitors have been assayed in vivo after having been fed to caterpillars in diet (Berenbaum and Neal, 1985; Hedin et al., 1988; Lindroth, 1989; Neal, 1989; Lindroth and Bloomer, 1991). However, feeding these enzyme inhibitors directly to insects in their food may present special problems, such as toxicity due to the inhibitor alone, enzyme induction by the inhibitor, or their metabolism by detoxifying enzymes.

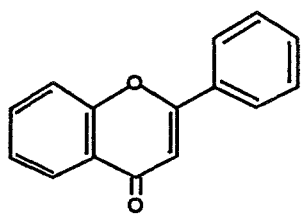

FIG. 1. The structure of flavone.

Second, preexposure of larvae to a subeffective dose of an allelochemical that induces detoxifying enzymes should allow larvae to better tolerate a diet containing a higher concentration of the same allelochemical. Thus, for two days we fed larvae an inducing diet containing flavone at a concentration that would induce detoxifying enzymes but not reduce growth, and then switched the larvae to a diet with a flavone dose that would reduce performance in uninduced larvae. Our hypothesis was that the performance of the preexposed (induced) larvae would be superior to that of the uninduced larvae that were switched directly from a control diet to the performance-reducing diet.

Before starting these experiments, we had to determine: (1) the degree of enzyme inhibition when caterpillars were fed the insecticide synergists; (2) the dose of flavone that induced enzyme activity but caused minimal reduction in caterpillar growth; (3) an effective dose of flavone (i.e., a dose that significantly reduced caterpillar growth); and (4) the extent of enzyme inhibition occurring at these allelochemical concentrations.

METHODS AND MATERIALS

Chemicals. The chemicals used in this study are listed below with the abbreviations and vendors' names in parentheses: aldrin, piperonyl butoxide (PB), and *S,S,S*-tributyl phosphorotrithioate (DEF) (Chem Service Inc., Westchester, Pennsylvania); triphenyltin chloride (TPC) (Aldrich Chemical Company, Milwaukee, Wisconsin); 1-chloro-2,4-dinitrobenzene (CDNB), diethyl maleate (DEM), ellagic acid, flavone (>99% purity by TLC), α-naphthyl acetate, quercetin, and reduced glutathione (Sigma Chemical Company, St. Louis, Missouri), and tri-ortho-tolyl phosphate (TTP) (Eastman Kodak Company, Rochester, New York). All other chemicals were purchased from commercial suppliers.

Insect Growth and Performance. Eggs were obtained from the USDA-ARS Insect Attractants and Basic Biology Laboratory, Gainesville, Florida. Larvae were reared en masse on a modified artificial diet (lacking all preservatives except sorbic acid; Greene et al., 1976) until the beginning of each experiment, at which time they were transferred to individual 30-ml clear plastic cups. All larvae were reared at 27 ± 1°C, 50 ± 15% relative humidity with a 14:10 light–dark photoperiod.

Insect performance was assessed by the calculation of nutritional indices (Waldbauer, 1968; Slansky and Scriber, 1985). Larvae were reared from the late third to early fourth instars [15–25 mg fresh mass (fm); hereafter referred to as mid-instars] through to the pupal stage on the experimental diets. Pupae were frozen and then dried (60°C) for two days in a convection oven before weighing (±0.1 mg). Initial dry biomass was estimated by comparing the mean

mass of a cohort ($N = 10$) of larvae weighed fresh and after drying for two days. Initial dry biomass of the larvae for the preexposure experiment (see "Benefit of Preexposure to Flavone" below) was assessed similarly except that cohorts of mid-instar larvae were reared for two days on each treatment diet to assess biomass at the time larvae were switched. Body mass relative growth rate [RGR; dry biomass gain (mg)/mean dry biomass (mg)/day], body mass relative consumption rate [RCR; dry mass consumed (mg)/mean dry biomass (mg)/day], the efficiency of converting digested food to insect biomass [ECD; biomass gain/(food ingested-feces)], and the approximate digestibility [AD; (food ingested-feces)/food ingested)] were calculated on a dry mass basis from the time the larvae were switched until pupation. For calculation of RGR and RCR, we followed Gordon (1968) in using the exponential mean biomass during the experiment, except that we used dry instead of live biomass.

Treatment Diets. Inhibitors were dissolved in a minimum of acetone, alphacel (ICN Biochemicals Inc., Cleveland, Ohio) was added (1% fm; acetone and alphacel alone for the control), and the mixture was rotoevaporated to dryness at 60°C. This mixture was then added to freshly prepared diet as it cooled. Additionally, enzyme-inducing diets containing flavone, synergism diets containing one or more enzyme inhibitors with flavone, and control diets lacking both synergist and flavone were prepared similarly.

Effects of Enzyme Inhibitors and Flavone on Larval Growth and Survival. The effect of each enzyme inhibitor or flavone on the mortality and RGR of caterpillars fed experimental diets was assessed. Flavone and each synergist were tested at a range of concentrations (Table 1).

Enzyme Activity of Larvae Fed Enzyme Inhibitors and Flavone. Enzyme activity was determined for larvae fed a range of enzyme inhibitor concentrations or flavone. Depending upon the experiment, either mid-instar ($N = 25$) or recently molted sixth-instar larvae ($N = 15$) were fed a test diet for two days, after which their midguts were removed and washed in ice cold (0–4°C) 1.15% KCl. Midguts were homogenized, and enzyme activity was measured in duplicate using standard techniques. Polysubstrate monooxygenase activity was measured by the aldrin epoxidase method (Yu, 1982). Samples comprising the crude homogenate as the enzyme source were incubated for 15 min at 30°C, and the amount of dieldrin produced was extracted with hexane and measured by gas chromatography on a 122 cm × 2 mm ID glass column packed with 3% SE 30 on 100/120 Gas Chrom Q. General esterase activity was measured by the α-naphthyl acetate method, using the crude homogenate as an enzyme source (Van Asperen, 1962; Yu et al., 1984). The amount of hydrolysis product (α-naphthol) was measured spectrophotometrically (600 nm) against a boiled blank. Glutathione transferase activity was measured by detecting CDNB conjugation with glutathione (Habig et al., 1974; Yu, 1984) using the postmitochondrial fraction as the enzyme source. The change in absorbance (340 nm) of the

TABLE 1. MORTALITY, RELATIVE GROWTH RATE (RGR), AND ENZYME ACTIVITY (MEAN ± SE) OF *S. fruqiperda* LARVAE FED INHIBITORS OF DETOXIFYING ENZYMES

Enzyme system/inhibitor	Concentration (% fm)	Mid-instar to prepupa: Mortality (%)	Mid-instar to prepupa: RGR (mg/mg/day)	Mid-instar to prepupa: N	Sixth instar: Enzyme activity (% of control)	Sixth instar: N
Control		0	0.46 ± 0.01	55	100[a]	
PSMO						
Piperonyl butoxide (PB)	0.01	0	0.42 ± 0.02	15	69.2 ± 10.4[b]	6
	0.05	0	0.44 ± 0.02	15	40.5 ± 3.1[b]	7
	0.1	0	0.41 ± 0.02	15	68.9 ± 13.0[b]	7
GE						
Triorthotolyl phosphate	0.01	0	0.43 ± 0.01	15	0.3 ± 0.3[b]	6
(TTP)	0.05	0	0.40 ± 0.02[b]	15	4.2 ± 3.3[b]	4
	0.1	0	0.36 ± 0.02[b]	15	0 ± 0[b]	4
S,S,S-tributyl	0.002	13.3	0.47 ± 0.02	13	4.8 ± 4.8[b]	5
phosphorotrithioate	0.004	0	0.40 ± 0.01	15	0.9 ± 0.7[b]	6
(DEF)	0.006	0	0.42 ± 0.01	15	0 ± 0[b]	2
	0.01	0	0.29 ± 0.02[b]	15	[c]	
	0.05	40.0	0.16 ± 0.02[b]	9		
	0.1	93.3		[d]		
GST						
Diethyl maleate (DEM)	0.01	0	0.46 ± 0.02	15	110.8 ± 8.5	6
	0.05	0	0.41 ± 0.02	15	178.7 ± 10.4	6

	0.1	0	0.47 ± 0.02	15	251.4 ± 30.4	8
	0.15		[c]		265.7 ± 26.6[b]	8
	0.2				362.3 ± 50.0[b]	5
	0.25				522.3 ± 116.3[b]	5
Triphenyltin chloride[f]	0.0004				111.8 ± 18.2	6
(TPC)	0.0005	6.7	0.39 ± 0.01[b]	14		
	0.0006				162.5 ± 12.4	5
	0.0008				139.8 ± 12.5	7
	0.001	13.3	0.15 ± 0.02[b]	5	214.9 ± 29.6	7
	0.0025				179.3 ± 10.3	4
	0.005	100			347.5 ± 144.3[b]	3
Quercetin	0.05	0	0.46 ± 0.01	15		
	0.1	0	0.43 ± 0.01	15	92.6 ± 6.0	5
	0.15	0	0.46 ± 0.01	15		
	0.2	0	0.41 ± 0.02	15	101.8 ± 9.3	5
	0.3				106.1 ± 8.5	5
Ellagic acid[g]	0.1	6.7	0.37 ± 0.02	14	102.0 ± 8.3	5
	0.2	0	0.30 ± 0.01[b]	15	87.0 ± 4.3	4
	0.3	0	0.31 ± 0.01[b]	15	97.8 ± 5.1	4

[a] Mean control activity differed for each enzyme system measured; see Results for values.
[b] Means differ significantly ($P = 0.05$) from the control.
[c] Larvae were used only for mortality and RGR, therefore no enzyme activity data are given.
[d] Insufficient number larvae ($N = 1$) survived to analyze data.
[e] Larvae were used only for enzyme assays, therefore no mortality or RGR data are given.
[f] This compound was also tested at 0.01, 0.05, and 0.1% fm; however, all larvae died at these concentrations.
[g] Ellagic acid was analyzed independently of other compounds. Means compared with control = 0.37 (±0.02) mg/mg/day.

incubation mixture was monitored for 2 min at 25°C. Furthermore, the GST inhibitors were analyzed in vitro by adding each compound directly to the incubation mixture. Ellagic acid is insoluble in the GST buffer (0.1 M, $NaPO_4$, pH 6.5) and therefore was dissolved in 20 μl NaOH (0.1 M), whereas the remaining compounds were dissolved in GST buffer. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

Synergism. A series of five experiments was conducted to assess the occurrence of synergism when flavone was combined at several concentrations with each enzyme inhibitor. Each experiment consisted of a single flavone concentration (0.02, 0.04, 0.08, 0.125, or 0.25% fm) combined with each enzyme inhibitor (PB 0.01, TTP 0.01, DEF 0.002, DEM 0.01, or TPC 0.0004% fm). A combined inhibitor treatment also was included (0.04, 0.08, 0.125, and 0.25% fm flavone only) that targeted all three enzyme systems, consisting of three inhibitors (PB 0.01, TTP 0.01, and DEM 0.01% fm). Control diets for each experiment were: (1) flavone-free and inhibitor-free; (2) flavone alone; and (3) each inhibitor alone. Caterpillar mortality and RGR were recorded from the mid-instar through to the pupal stage. Significant synergism occurred if the reduction in RGR due to the synergized treatment (flavone + inhibitor) was significantly greater than the added reduction due to the flavone-alone and the inhibitor-alone controls. Additionally, PSMO, GE, and GST activities were measured (as described above) for each combined flavone (0.02% fm)–synergist treatment.

Benefit of Pre-exposure to Flavone. An experiment was conducted to determine whether pre-exposure to a flavone concentration that induced detoxifying enzymes yet had a negligible negative impact on insect performance would benefit larvae subsequently challenged with an effective flavone concentration that reduced RGR in uninduced individuals (see Results). Following a two-day pre-exposure period, during which larvae (15–23 mg fm) were fed a diet with 0.02% flavone, they were switched to a diet with 0.125% flavone (induced larvae). Other treatments consisted of larvae reared on a control diet for two days and then switched to another control diet (control larvae), and larvae switched to the 0.125% flavone diet directly from the control diet (uninduced larvae). The entire experiment was replicated three times.

Statistical Analyses. Mortality data were analyzed by a G test of independence (Sokal and Rohlf, 1981), whereas all remaining analyses were conducted with PC/SAS (SAS Institute, 1987). To determine the inhibitor levels that did not significantly reduce RGR, the effect of these compounds was analyzed with a one-way analysis of variance (ANOVA) followed by a Dunnett's test ($P = 0.05$) comparing each treatment mean with the appropriate control mean. The effect of flavone concentration on RGR was determined by linear regression. The enzyme activity data were also analyzed by one-way ANOVA

followed by the Dunnett's test. The results from the synergism experiments were analyzed with one-tailed Student's t-test comparisons to test whether the synergist reduction in RGR (i.e., each synergist–flavone combination) was significantly greater than the additive reduction in RGR due to flavone and each synergist alone. The results of the preexposure experiments were analyzed by a two-way ANOVA with interaction, where the main effects were the three experiments and the three treatments. If the interaction was not significant, the data for the experiments were combined. One-tailed orthogonal contrasts were made between: (1) the control and the combined effects of the uninduced and the induced treatments, to determine whether dietary flavone reduced caterpillar performance; and (2) the uninduced versus the induced treatments, to determine if performance of the induced larvae improved relative to that of the uninduced insects.

RESULTS

Effects of Enzyme Inhibitors and Flavone on Larval Growth and Survival. Caterpillar sensitivity to dietary enzyme inhibitors differed greatly among inhibitors. The PSMO inhibitor PB had no effect on RGR or survival at any concentration tested (Table 1). All larvae fed the GE inhibitor TTP survived but their RGR values were reduced at the highest concentrations compared with controls. Larvae fed the other GE inhibitor, DEF, had both greater mortality and reduced RGRs at the highest concentrations. Feeding the GST inhibitor, DEM, resulted in no mortality or reduction in RGR at any concentration tested. However, all larvae died when fed the other GST inhibitor, TPC, at concentrations equal to or greater than 0.005% (0.01, 0.05, 0.1% data not shown). Although mortality was low at 0.0005 and 0.001% TPC, these concentrations significantly reduced RGR. Mortality was low when caterpillars were fed either quercetin or ellagic acid, but there were significant reductions in RGR at ellagic acid concentrations greater than 0.1%.

Flavone significantly reduced RGR with increased concentration (Figure 2). No larvae died when fed diets containing flavone concentrations less than or equal to 0.25%, whereas all died at 0.50 and 0.75% fm.

Enzyme Activity of Larvae Fed Enzyme Inhibitors. Feeding larvae the PSMO inhibitor PB significantly reduced PSMO activity to less than 70% of the control (173.0 $\pm$ 6.0 pmol/min/mg protein) at all concentrations tested (Table 1). General esterase activity was significantly reduced to less than 5% of the control activity (423.7 $\pm$ 81.1 nmol/min/mg protein) by dietary TTP and DEF at all concentrations tested. Inhibition of either of these enzyme systems did not increase with increased concentration of the inhibitors. In contrast, GST activity increased significantly in larvae fed the highest concentrations of DEM (0.15–

0.25%) and 0.005% TPC; maximum activity was 5.2- and 3.5-fold greater, respectively, than the control (365.9 ± 22.6 nmol/min/mg protein). Considerable variation occurred in GST activity at the highest TPC concentration (0.005% fm) tested. No protein was detected in extracts from two of the five TPC experiments conducted, and the data therefore were omitted from the analysis. GST activity was not influenced by quercetin or ellagic acid at any concentration tested. Analysis of in vitro GST incubations that included the inhibitors (10^{-2} M) indicated that under these conditions all synergists tested except DEM significantly reduced enzyme activity (Table 2). Addition of 20 μl of the ellagic acid carrier (0.1 M NaOH) alone did not significantly alter GST activity (85.1 ± 2.3% of control; $P > 0.05$).

Enzyme Activity of Larvae Fed Flavone. All three enzyme systems increased in activity when the mid-instar larvae were fed 0.02% flavone (Table 3). The PSMO, GE, and GST systems had significantly greater activities ($F = 25.6$, *df*

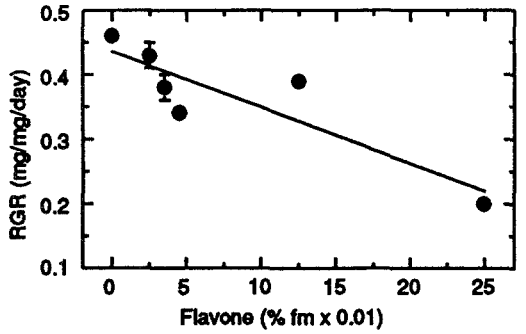

FIG. 2. Relative growth rates (RGR; mean ± SE) of *S. frugiperda* mid-instar larvae fed diets with different flavone concentrations [$R^2 = 0.55$; $P < 0.0001$; RGR = 0.44 − 0.87(flavone concentration)]. Each data point represents the mean of 15 larvae.

TABLE 2. IN VITRO INHIBITION OF GLUTATHIONE TRANSFERASE (GST) ACTIVITY (MEAN ± SE) OF MIDGUT PREPARATIONS FROM SIXTH-INSTAR *S. frugiperda*

Enzyme inhibitor (10^{-2} M)	Enzyme activity (% of control)	N^a
DEM	89.1 ± 9.3	4
TPC	2.3 ± 0[b]	2
Quercetin	2.3 ± 1.6[b]	2
Ellagic acid	5.8 ± 0.8[b]	2

[a] Number of experiments where each assay was conducted in duplicate.
[b] Means differ significantly ($P = 0.05$) from the control.

TABLE 3. ENZYME ACTIVITY (MEAN ± SE) OF MID-INSTAR *S. frugiperda* LARVAE FED FLAVONE (0.02% fm)

Enzyme[a]	Specific activity (nmol/min/mg protein)[b]	
	Control	Flavone
PSMO	93.5 ± 8.8[c]	257.1 ± 31.1[c,d]
GE	383.7 ± 59.9	576.8 ± 108.3[d]
GST	183.4 ± 47.6	319.4 ± 9.8[d]

[a] Polysubstrate monooxygenase (PSMO); general esterase (GE); and glutathione transferase (GST).
[b] Means obtained from six experiments, five for GE/flavone, where each assay was conducted in duplicate.
[c] pmol/min/mg protein.
[d] Significantly different from the control ($P = 0.05$, see text).

$= 1,11$, $P = 0.0005$; $F = 14.31$, $df = 1,10$, $P = 0.016$; and $F = 91.88$, $df = 1,11$, $P < 0.0001$, respectively), that were 2.8-, 1.5- and 1.7-fold greater than the controls, respectively.

These activities also increased in sixth-instar larvae fed flavone over that of the flavone-free control. PSMO activity increased significantly at all flavone concentrations (Figure 3A). GE activity increased significantly only at the highest flavone concentration tested (0.25%) (Figure 3B). GST activity of larvae fed flavone at all concentrations was significantly greater than that of the control (Figure 3C). The PSMO system was the most inducible, with a 3.5-fold increase over the control; the GE and GST systems increased 1.5- and 3-fold, respectively.

Synergism. Larval mortality was very low (0–2.5%) in four of the five experiments; only in the flavone 0.25% experiment was there substantial mortality. Although no larvae died in the flavone- and synergist-free controls in this experiment, mortality for those treatments that included flavone at the 0.25% level ranged from 27% to 60% and was independent of the treatments ($P > 0.1$). Because of the substantial mortality, this experiment (0.25% flavone) was not included in the remaining analyses.

Consistent with the results reported above (Figure 2), RGR decreased with flavone concentrations greater than 0.02% (Figure 4). Additionally, significant reductions in PSMO (PB: $F = 53.27$; $df = 1,26$; $P < 0.0001$) and GE (TTP: $F = 24,701.36$; $df = 1,7$; $P < 0.0001$; and DEF: $F = 37,664.12$; $df = 1,7$; $P < 0.0001$) enzyme activities occurred when larvae were fed combinations of each enzyme inhibitor and flavone (0.02% fm; Table 4), similar to each inhibitor alone (Table 1). Furthermore, GST activity increased significantly when larvae were fed combinations of flavone (0.02% fm) and DEM ($F = 14.75$; $df = 1,6$;

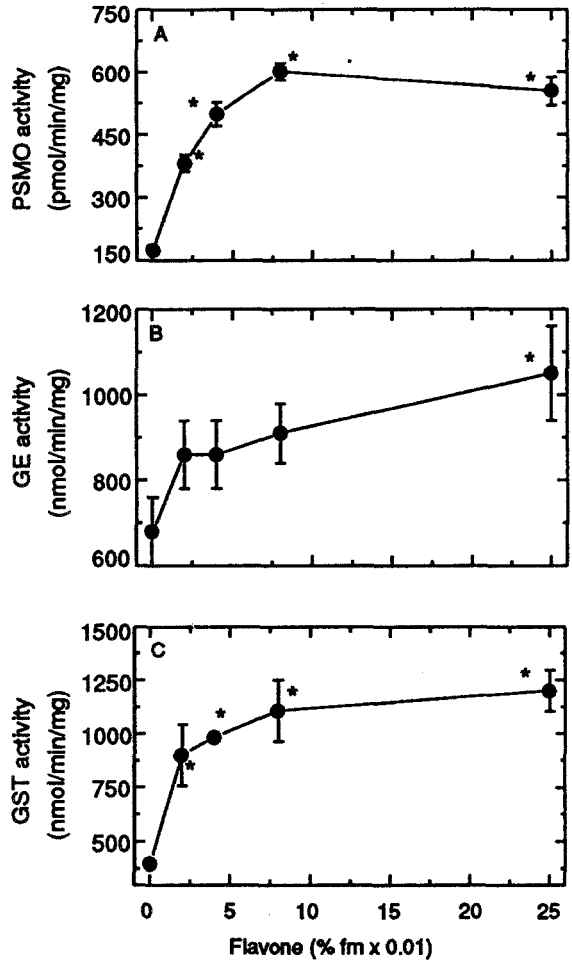

FIG. 3. Polysubstrate monooxygenase (A), general esterase (B), and glutathione *S*-transferase (C) activity (mean ± SE) of *S. frugiperda* sixth-instar larvae fed diets with different flavone concentrations. Each data point represents the mean of four to eight experiments, where each assay was conducted in duplicate. An asterisk next to a data point indicates a significant difference ($P = 0.05$) from the flavone-free control.

$P = 0.009$) or flavone and TPC ($F = 366.75$; $df = 1,7$; $P < 0.0001$), as occurred when flavone alone was fed to larvae (Figure 3). These increases in GST activity were probably due to flavone and not the inhibitors, because at these concentrations neither DEM (0.01) nor TPC (0.0004% fm) significantly altered GST activity (Table 1).

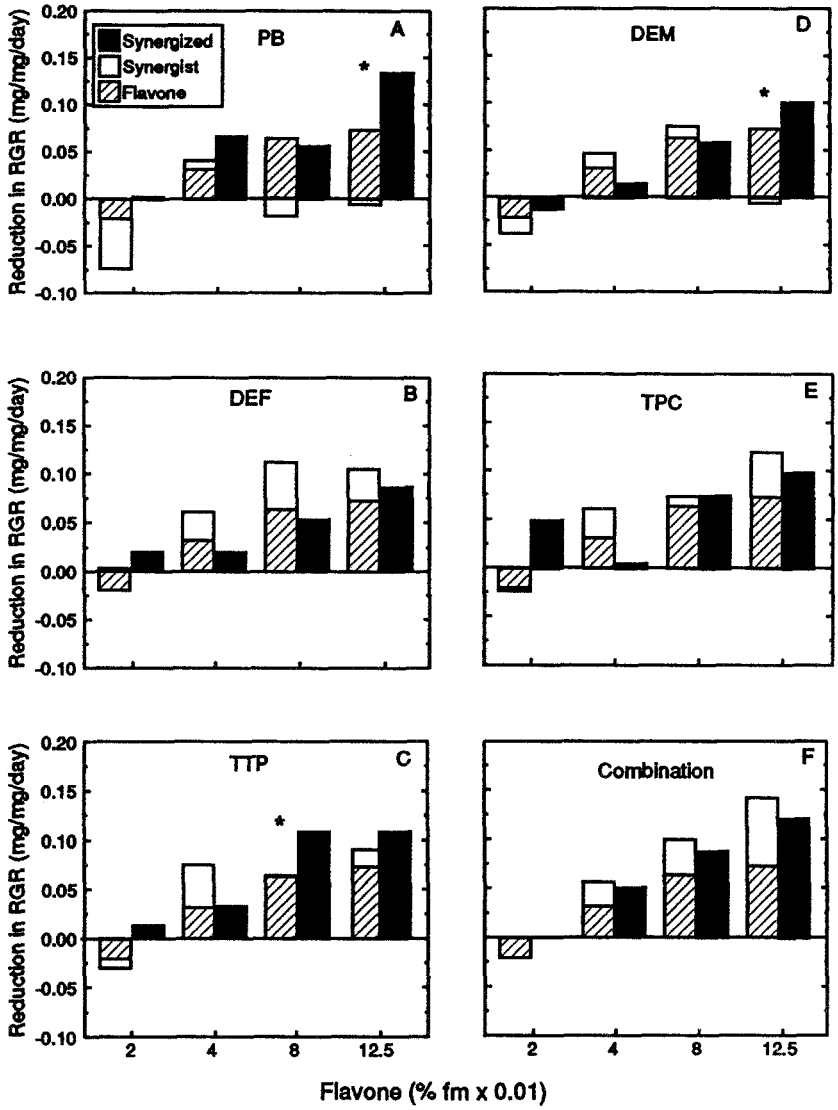

FIG. 4. Reductions in mean RGR for *S. frugiperda* larvae fed diets containing either flavone, an enzyme inhibitor, or flavone plus each enzyme inhibitor. The inhibitors used were piperonyl butoxide (PB); tri-ortho-tolyl phosphate (TTP); *S,S,S*,-tributyl phosphorotrithioate (DEF); diethyl maleate (DEM); and triphenyltin chloride (TPC). The combination treatment consisted of diet containing three inhibitors (PB, TTP, and DEM). Flavone was tested at four concentrations. Significant synergism (indicated by an asterisk) occurred when the reduction in RGR of the synergized treatment was significantly greater than that of the additive effect of flavone and the enzyme inhibitor (indicated by the height of these bars combined). Each bar represents the mean of 15 larvae except for the following treatments: $N = 14$ for flavone and synergized TPC at 0.02%, DEF and synergized TTP at 0.04%, PB, the combination and the synergized PB at 0.08%, DEF at 0.125%, and $N = 11$ for the synergized combination treatment at 0.125% flavone.

TABLE 4. PERCENT OF FLAVONE-FREE CONTROL (MEAN ± SE) ENZYME ACTIVITY OF SIXTH-INSTAR *S. frugiperda* FED FLAVONE (0.02% fm) AND EACH ENZYME INHIBITOR

Enzyme system[a]	Inhibitor (% fm)	Activity (% of control)	N^b
PSMO	PB (0.05)	56.1 ± 4.8[c]	6
GE	TTP (0.05)	5.7 ± 1.4[c]	2
	DEF (0.004)	4.4 ± 1.2[c]	2
GST	DEM (0.01)	261.7 ± 41.8[c]	2
	TPC (0.0004)	256.0 ± 6.4[c]	2

[a] Enzyme systems: Polysubstrate monooxygenase (PSMO); general esterase (GE); and glutathione transferase (GST). Inhibitors: piperonyl butoxide (PB); triorthotolyl phosphate (TTP); *S,S,S,*-tributyl phosphorotrithioate (DEF); diethyl maleate (DEM); triphenyltin chloride (TPC).
[b] Number of experiments, each assay conducted in duplicate.
[c] Significantly different ($P = 0.05$) from the controls (see text).

Significant synergism was found when PB, TTP, and DEM were combined with the higher flavone concentrations. PB and DEM exhibited synergism at 0.125% fm flavone, whereas TTP synergized flavone at 0.08% (Figure 4). However, no significant synergistic effects were found at 0.02 and 0.04% flavone. Furthermore, the inhibitors DEF, TPC, and the combination treatment did not significantly synergize flavone at any concentration tested. The RGR increased slightly, as indicated by the negative bars in Figure 4, when larvae were fed the lowest dose of flavone (0.02%) or several of the synergists alone.

Benefit of Pre-exposure to Flavone. Significant interactions between the main effects (study and treatment) occurred only with RCR ($F = 4.06$; $df = 4,120$; $P = 0.004$); thus, only this performance index was analyzed separately for each study. Two uninduced and four induced larvae died after the dietary switch but before the prepupal stage; however, these differences were independent of the treatments ($P > 0.05$). Biomass of uninduced and induced larvae after the two-day preexposure period was not significantly ($P = 0.17$) different from the control, whereas biomass was significantly reduced in the induced compared with the uninduced treatments ($F = 4.58$; $df = 1,129$; $P = 0.034$; Table 5). Biomass gained following the dietary switch significantly decreased in the uninduced and induced treatments ($F = 34.7$; $df = 1,126$; $P < 0.0001$) compared with the control; however, there was no significant difference between the uninduced and induced larvae. RGR significantly decreased in the uninduced and induced treatments compared with the control ($F = 156.50$; $df = 1,126$; $P < 0.0001$) and, furthermore, RGR of the uninduced larvae was significantly reduced compared with the induced larvae ($F = 6.68$; $df = 1,126$; $P = 0.035$). No significant changes in AD occurred for any of the treatments. ECD decreased

TABLE 5. NUMBER OF *S. frugiperda* LARVAE SURVIVING, THEIR MEAN (±SE) BIOMASS AFTER BEING FED FOR TWO DAYS ON INDUCING DIET, BIOMASS GAIN, RELATIVE GROWTH RATES (RGR), APPROXIMATE DIGESTIBILITY (AD), AND EFFICIENCY OF CONVERSION OF DIGESTED FOOD TO BIOMASS (ECD) AFTER PRE-EXPOSURE

Treatment	N^a	Mean initial biomass (mg fm)	Biomass gain (mg)	RGR (mg/mg/day)	AD (%)	ECD (%)
Control	45/45	73.4 (±2.8)NS[b]	30.1 (±1.1)*	0.23 (±0.01)*	54.2 (±1.1)NS	31.8 (±1.0)*
Uninduced	45/43	73.0 (±3.0)	22.2 (±1.1)	0.14 (±0.01)	51.7 (±1.3)	28.0 (±1.1)
Induced	45/41	64.9 (±2.2)*[c]	23.0 (±0.8)NS	0.16 (±0.01)*	52.3 (±1.4)NS	29.4 (±1.3)NS

[a] Number surviving the pre-exposure treatment/number surviving to prepupal stage.
[b] Control means followed by an asterisk (NS = not significant) are significantly greater than the uninduced and the induced treatments by a one-tailed orthogonal contrast (see Methods and Materials).
[c] Induced means followed by an asterisk (NS = not significant) are significantly different from the uninduced means by a one-tailed orthogonal contrast (see Methods and Materials).

only for the uninduced and induced treatments compared with the control larvae ($F = 10.22$; $df = 1,126$; $P = 0.01$; Table 5). RCR decreased significantly in the uninduced and induced treatments compared with the flavone-free control in experiments 1 ($F = 38.46$; $df = 1,42$; $P < 0.0001$), 2 ($F = 20.66$; $df = 1,42$; $P < 0.0001$), and 3 ($F = 15.94$; $df = 1,38$; $P = 0.0003$). Additionally, the induced larvae had significantly greater RCR values compared with the uninduced larvae only in experiments 1 and 3 (Figure 5).

DISCUSSION

Our experiments were designed to test the importance of detoxifying enzyme activity in the ability of larvae of the polyphagous *S. frugiperda* to tolerate ingested flavone. Little, if any, biologically significant benefit resulted from uninhibited constitutive enzyme activity or from pre-exposure to inducing doses of flavone. If the level of in vitro PSMO activity measured here is representative of the activity of caterpillar midgut tissues in vivo, and if flavone is detoxified by these enzymes, then the inhibition observed with PB (approximately 50%) should effectively double the concentration of flavone in the insect. However, combining PB and flavone in the diet resulted in significant synergism only at the highest flavone concentration tested (0.125%; Figure 4A). Furthermore, a more than two-fold increase in PSMO activity following pre-exposure did not dramatically benefit larvae subsequently exposed to a growth-reducing concentration of flavone (Table 5, Figure 5).

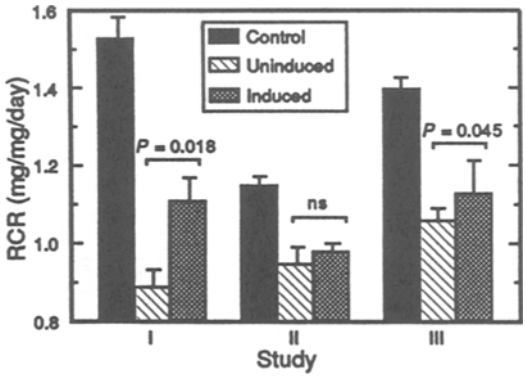

FIG. 5. Relative consumption rates (RCR) (mean ± SE) of *S. frugiperda* larvae fed either control or flavone (0.02%) diets for two days, then switched to a control (control or uninduced) or a flavone (0.125%; induced) diet and reared to pupation. The statistical comparisons indicated are one-tailed orthogonal contrasts between the uninduced and induced treatments.

Synergism. The results of the synergism studies varied considerably and therefore should be interpreted with caution. We predicted that flavone would be metabolized by ring hydroxylation by the PSMO system, a common mode of action for this enzyme system (Hodgson et al., 1991), and that inhibiting this system with PB would have a greater negative impact on caterpillar performance than inhibition of the GE and GST enzyme systems. Although the GE inhibitors were almost 100% effective, we did not expect this inhibition to affect flavone metabolism because this compound lacks the active site (an ester bond; Figure 1) for these enzymes. As none of the GST inhibitors reduced GST activity in vivo, synergism of flavone was not expected, even if these enzymes were involved in flavone detoxification. However, in addition to the effect of PB, significant synergism occurred when flavone diets contained the GE inhibitor TTP or the GST inhibitor DEM (Figure 4C and D, respectively). Synergism by DEM may have occurred because DEM inhibited PSMO activity, as found in vitro in house flies (Welling and DeVries, 1985), although the effect of DEM on PSMO activity was not determined in our experiments. The reason for synergism with TTP and flavone (0.08%; Figure 4C) is unknown. Furthermore, in several of our experiments with various inhibitors (Figure 4B–E), the additive reduction in RGR resulting from flavone and the synergist administered singly was much greater than that of the synergized treatment; the cause of these results is also unknown. Finally, synergism with flavone was not significant in any of the combination treatments, in which an inhibitor of each enzyme system was combined, even though synergism occurred when these same inhibitors were used individually. Possibly the synergists in the combination treatments antagonized one another. Thus, even though we found one example of synergism that fit our prediction (i.e., PB; Figure 4A), many of our results remain unexplained.

Synergism may result when the co-application (usually topical) of an enzyme inhibitor and a toxic pesticide causes a significantly greater reduction in insect performance or an increase in mortality compared with the additive effect of each component in the mixture (Metcalf, 1967). Feeding synergists to caterpillars prolongs exposure to the compounds and thus represents a different situation from that of a single topical dose. Thus, detoxifying enzymes may be induced, possibly resulting in increased metabolism of the synergist (Yu and Terriere, 1974). However, these inhibitors (PB, TTP, DEF, and DEM) have been used successfully to synergize allelochemicals when fed to insect larvae (Hedin et al., 1988; Lindroth, 1989). Furthermore, other workers have reported synergism when larvae of *Heliocoverpa zea* and *Depressaria pastinacella* were fed the allelochemical xanthotoxin with PB or other naturally occurring methylenedioxyphenyl-containing inhibitors (Berenbaum and Neal, 1985; Neal, 1989; Lee and Berenbaum, 1990).

The levels of in vivo PSMO inhibition with PB found in our study were

similar to those of 1-day-old house fly (Yu and Terriere, 1974) and sixth-instar *S. frugiperda* larvae fed PB (Yu and Hsu, unpublished data). Furthermore, they are in the range of previous reports of in vitro inhibition, ranging from 30% to 93% (e.g., Yu, 1987; Nitao, 1989). We found the greatest in vivo inhibition with the GE inhibitors, results that are similar to in vitro analyses reported previously (Yu, 1990). In contrast, although each GST inhibitor except DEM significantly reduced GST activity when added to the in vitro midgut preparations in our study, they did not reduce in vivo GST activity. Instead, the dietary GST inhibitors DEM and TPC induced GST activity, as is often found with dietary xenobiotics (Yu, 1986), including PB in house flies (Yu and Terriere, 1974) and black cutworm larvae (Thongsinthusak and Krieger, 1974). Possibly this biphasic activity occurred in response to DEM and TPC, where GST enzyme inhibition occurred initially, followed by enzyme induction (Yu and Terriere, 1974; Thongsinthusak and Krieger, 1974). However, we are unaware of this biphasic response occurring in insects exposed to any inhibitor other than PB. Induction of detoxifying enzyme activity suggests that the metabolism of these inhibitors may be increased. Neither quercetin nor ellagic acid significantly influenced GST activity, although, as was found in human and rat liver preparations (Das et al., 1986), both compounds (and TPC) reduced in vitro GST activity.

The failure of DEM to inhibit GST enzyme activity in vitro was unexpected, as this is a commonly used insecticide synergist (e.g., Argentine et al., 1989; Pree et al., 1990). Several reasons for this apparent lack of inhibition can be proposed. The use of DEM is based on reduced (by about 50% after 2 hr) GST activity of house fly larvae after topical application (Welling and DeVries, 1985). In mammals, DEM reduces GST activity by depleting the supply of glutathione (see references in Welling and DeVries, 1985). However, in house flies the decrease in glutathione concentration following DEM treatment was not sufficient to cause the observed reduction in enzyme activity (Welling and DeVries, 1985). Possibly, despite depletion of glutathione by DEM (as in mammals), enough glutathione remained in our assays to allow the reaction to proceed normally. Finally, Yu (1989) found six isozymic forms of *S. frugiperda* GST; thus it is possible that the GST isozyme responsible for CDNB conjugation was not sensitive to inhibition by DEM. Furthermore, individual isozymes of the GST system may have been inhibited, as reported by Welling and DeVries (1985), but the substrate we used may have been conjugated by a different GST isozyme. Analysis of individual GST isozymes and inhibition by DEM with additional substrates would be necessary to determine why we failed to find GST inhibition with DEM.

Benefit of Pre-exposure to Flavone. Although PSMO and GST enzyme activities in the flavone-fed (0.02%) larvae were 2.8- and 1.7-fold greater in the mid-instars and 2.2- and 2.3-fold greater in the sixth instars, respectively,

than in the controls, subsequent feeding on the effective-dose diet (0.125% flavone) improved RGR of the induced compared with the uninduced larvae by only 14%. Three possible reasons for this marginal benefit of enzyme induction are discussed here. First, maximum PSMO activity occurs about two to three days following exposure to an inducer (Brattsten and Wilkinson, 1973; Yu, 1982). Detoxifying enzymes in the uninduced larvae may have become induced after the switch to flavone (0.125%), permitting recovery from the toxic effect of ingested flavone. Slansky (unpublished data) found in similar pre-exposure experiments with another allelochemical that the results may depend upon when performance is measured after diet switch. However, induced *S. frugiperda* larvae, allowed to develop for just two days after the switch to the 0.125% flavone diet, did not have greater RGR or RCR values than uninduced larvae (data not shown), in contrast to larvae that were allowed to complete development to the pupal stage. Second, flavonoids (e.g., the pentahydroxy flavone quercetin) inhibit the superoxide dismutase antioxidant enzymes that protect several species of herbivores from superoxide anions (Pritsos et al., 1991). These highly reactive species may react deleteriously with macromolecules such as DNA, RNA, lipids, and proteins (Felton and Duffey, 1991). However, it is not known if flavone influences the antioxidant enzymes in *S. frugiperda*. Third, flavone tolerance or PSMO induction by flavone may have differed among generations of *S. frugiperda*. Both RGR and RCR for all treatments were lower in the second experiment, suggesting that the larvae in this experiment had a reduced ability to respond adaptively to flavone.

Our data showing a benefit of pre-exposure may be the result of habituation to a repellent diet. Both RCR and RGR increased in the induced larvae compared with the uninduced larvae. The improved RGR may simply reflect the greater RCR, rather than an increased tolerance to flavone. An increase in RCR after pre-exposure may be explained by desensitization of deterrent receptors (Szentesi and Jermy, 1990). In a subsequent choice test (Slansky and Wheeler, unpublished), however, flavone at 0.125% deterred *S. frugiperda* larvae, but the effect did not decrease following a two-day exposure to the 0.02% fm diet. Thus, desensitization is considered to be an unlikely explanation for these results.

Induction of detoxifying enzymes may be a generalized response to the presence of xenobiotics (Dowd, 1990). Although flavone induces all of the enzyme systems we investigated, as well as others (Yu, 1986), and is metabolized in vitro by the PSMO system (Yu, 1987), it may not be effectively detoxified in vivo. Similar to our results, Harwood et al. (1990) found that, although all of the peppermint monoterpenes they studied were metabolized in vitro by the PSMO system, apparent inhibition of this system with PB synergized only two of the four allelochemicals tested. Thus, in vitro metabolism may not always reflect in vivo metabolism. Furthermore, the potential flavone metabolites (possibly two to three) recovered from frass produced by uninduced larvae were

each equal in concentration to those produced by the flavone-induced larvae (Wheeler, unpublished data). These results suggest that in vivo metabolism of flavone occurs but the metabolite(s) may be as toxic as the parent compound or more so (Fukami et al., 1967; Isman et al., 1987; Koul et al., 1990). Overall, our findings suggest that, at best, constitutive and induced detoxifying enzymes may marginally enhance flavone tolerance in *S. frugiperda* larvae. However, more studies are needed evaluating the relationship between in vitro and in vivo detoxifying enzyme activity and the benefit of enzyme induction before general conclusions may be drawn regarding the role of detoxifying enzymes in allelochemical tolerance.

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