FOREST ENTOMOLOGY

Toxicity of Diflubenzuron in Larvae of Gypsy Moth (Lepidoptera: Lymantriidae): Effects of Host Plant

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J. Econ. Entomol. 86(3): 809–814 (1993)

ABSTRACT Larvae of gypsy moth, *Lymantria dispar* (L.), reared on Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, were significantly more tolerant of both topically and orally administered diflubenzuron than were those raised on white alder (*Alnus rhombifolia* Nutt.). Topical administration resulted in an LD_{50} of 8.96 μg/g larva for larvae reared on Douglas-fir and 3.58 μg/g larva for larvae reared on alder. The LC_{50}s obtained with orally administered diflubenzuron were 0.38 ppm for larvae reared on Douglas-fir and 0.07 ppm for larvae reared on alder. Topically treated females reared on either host and orally treated females reared on alder required significantly longer to pupate than did controls, but developmental periods of males reared on alder and both sexes reared on Douglas-fir were unaffected by oral treatment. Pupal weights were not affected by treatment in either sex, whether larvae were reared on Douglas-fir or alder.

KEY WORDS benzoylphenylureas, *Lymantria dispar*, host plant

DIFLUBENZURON (Dimilin), a benzoylphenyl urea, interferes with chitin synthesis (Cohen 1987). New cuticle is malformed and endocuticular layers are disrupted (Gijswijt et al. 1979, Grosscurt & Jongsma 1987, Hassan & Charnley 1987, Percy-Cunningham et al. 1987). Diflubenzuron also decreases food consumption and growth rate (Mulder & Gijswijt 1973, Ascher & Nemny 1976, Radwan et al. 1986). The effects of this compound become particularly obvious between treatment and ecdysis.

Diflubenzuron successfully controls larvae of the gypsy moth, *Lymantria dispar* (L.) (Granett & Dunbar 1975, Miller & West 1987), and is being implemented into integrated pest management programs for this major defoliator (Granett 1987). However, previous studies on toxicity of this compound to gypsy moth (Granett & Dunbar 1975, Granett & Weseloh 1975, Abdelmonem & Mumma 1981) have been conducted with larvae reared on artificial diet. Plant allelochemicals modify levels of detoxifying enzymes in herbivores and, therefore, their susceptibility to insecticides (Berry et al. 1980, Terriere 1984, Brattsten 1988, Lindroth 1989, Sheppard & Friedman 1989, Lindroth et al. 1990, Moldenke et al. 1992). Because metabolism appears to play a major role in the toxicity of diflubenzuron to insects (Pimprikar & Georghiou 1979, Retnakaran et al. 1985), the host plant consumed by a polyphagous insect such as gypsy moth could well affect its susceptibility to this insecticide. Virtually nothing is known, however, about the effects of host plants or their foliar constituents on toxicity of these compounds to leaf-eating insects.

We report here the results of studies on toxicity of diflubenzuron to third-instar gypsy moth feeding on Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, and white alder, *Alnus rhombifolia* Nutt. These plants were chosen because of their differential effects on larval development, with alder being the more suitable host (Miller & Hanson 1989a,b; Miller et al. 1991), and the presence of certain dominant allelochemicals. The dominant allelochemicals in Douglas-fir foliage are monoterpenes (Von Rudloff 1973, Joseph et al. 1991) and phenolics (Joseph et al. 1991). In white alder, the principal foliar allelochemicals are phenolics, particularly condensed tannins; monoterpenes are not present (Joseph et al. 1991). Monoterpenes are potent inducers of detoxifying enzymes in lepidopteran larvae (Brattsten 1986, Yu 1986, Harwood et al. 1990). Induction of detoxifying enzymes by phenolics is less well studied, but phenolics may also induce some detoxifying enzymes, particularly esterases (Lindroth et al. 1990, 1991).

Materials and Methods

Insects. Egg masses were obtained from an oak woodland in Maryland in January 1989 and held at 5°C until needed. Experiments were conducted from early June through late August of 1989.

At the start of each experiment, the protective hairs were removed from eggs from several

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masses by rolling them gently with fine sand. The eggs were then pooled and washed in 10% formalin for 10 min to eliminate possible virus contamination. Larvae were reared in 148-ml cups with lids (10 larvae per cup) at 24°C, 45–50% RH, and a photoperiod of 16:8 (L:D) h. Except during administration of oral diflubenzuron, larvae were fed fresh, field-collected foliage that had been washed in 5% Chlorox, rinsed with distilled water, and air-dried to remove surface water. Stems of foliage fed to larvae before treatment were kept in water in 6 by 50 mm test tubes to minimize changes in allelochemistry; foliage fed to older larvae after treatment was eaten so rapidly that keeping it in water was unnecessary. Larvae were always supplied with excess foliage.

Foliar Chemistry. The fresh foliage used to feed the larvae was subsampled each week and the foliar nitrogen, terpene, and phenolic contents were determined. Details of foliage analysis have been reported by Joseph et al. (1991) and Moldenke et al. (1992).

Douglas-fir needles used in terpene analysis were carefully detached from branches, pooled, and frozen in an air-tight plastic bag until analyzed. Terpenes were extracted from the needles and determined by gas chromatography and gas chromatography/mass spectrometry as described in Joseph et al. (1991). Only needles from the previous year's growth were used in these analyses.

Phenolic content was determined from subsamples of fresh-frozen foliage. Total phenols were extracted from the foliage and analyzed by the method of Julkunen-Tiitto (1985). Phenols were estimated in terms of catechin equivalents.

Nitrogen content was determined by a micro-Kjeldahl procedure after foliage had been washed, oven-dried (60°C, 48 h), and ground in a Wiley mill to pass a 20-mesh screen.

Bioassay of Topical Toxicity. Two days after molting, third instars were selected, weighed, and treated topically on the thoracic region. Larvae reared on alder were treated with one of nine doses of diflubenzuron (technical grade), ranging from 0.5 to 12 μg/g body weight. Those reared on Douglas-fir were treated with one of 10 doses, ranging from 0.5 to 16 μg/g body weight. We applied 1 μl solution/10 mg body weight, using a microapplicator (Shardlow Micrometers, Sheffield, England). Control larvae were treated with acetone only. In each of three experiments, 15 larva were treated with each dose; each dose was replicated three times.

Treated larvae were replaced on their respective host plants in 148-ml covered cups immediately after treatment and observed daily. Mortality was recorded when larvae failed to molt and did not respond to probing. After 2 d, survivors were transferred to clean 148-ml cups, fed fresh alder or Douglas-fir foliage until pupation, and kept until death or adult emergence. Pupal weight and days required to complete development were recorded for all survivors as indicators of possible sublethal effects of treatment.

Bioassay of Oral Toxicity. Oral toxicity of diflubenzuron on third instars was determined by incorporating diflubenzuron into a diet of alder or Douglas-fir foliage (ground in liquid N_2 to pass through a 20-mesh screen) bound with agar. Agar (7.5 g agar/250 ml water for the Douglas-fir diet; 12.5 g agar/250 ml water for the alder diet) was dissolved, cooled to 60°C, and mixed with 36.25 g of foliage and the appropriate dose of diflubenzuron.

Two days into the third stadium, larvae were treated with one of seven doses of diflubenzuron in acetone, ranging from 0.0625 to 4 ppm of diet, in the diet corresponding to their original foliage type. After feeding on the treated diet for 48 h, larvae were transferred to their original foliage type, which they received until death or pupation. Mortality was recorded when larvae failed to molt and did not respond to probing. Subacute effects were determined for surviving larvae by measuring days for larval development and pupal weights.

Data Analyses. Diflubenzuron topical (LD_{50}) and oral (LC_{50}) toxicities on third instars fed different host plants were determined by probit analysis with POLO (Probit Or LOgit Analysis), a computer program developed by Russell et al. (1977). The criterion for significant differences between LD_{50}s and LC_{50}s was failure of the 95% confidence limits to overlap. Regression analysis and the chi-square test for goodness-of-fit were used to fit data to probit lines. The likelihood ratio tests described by Savin et al. (1977) were used to test for equality and parallelism of the probit lines. Analysis of variance was used to determine significance among the development parameters measured, and a Student's *t* test was used to determine significance between diets.

Results

Toxicity of Diflubenzuron. Larvae reared on Douglas-fir were significantly more tolerant of both topically and orally administered diflubenzuron than were those raised on alder (Table 1). The LD_{50} of topically applied diflubenzuron on larvae reared on Douglas-fir was more than twice that for larvae reared on alder. The LC_{50} for third instars receiving diflubenzuron orally in the Douglas-fir-based diet also was significantly higher than that for those receiving the compound in the alder-based diet. The probit regression lines for larvae fed Douglas-fir or alder, treated topically or orally with diflubenzuron, were parallel but unequal.

In both the topical and the oral treatment, larvae receiving diflubenzuron lived several days after treatment but died while attempting to molt

Table 1. Toxicity of topically and orally administered diflubenzuron after 48 h to third-instar gypsy moth reared on alder or Douglas-fir

Administration/diet	n	Slope ± SE	LD_{50} (95% CI) or LC_{50} (95% CI)	χ^2
Topical (LD_{50} μg/g larva)				
Alder	450	1.67 ± 0.15	3.58 (2.8 – 4.4)	6.9
Douglas-fir	495	1.27 ± 0.21	8.96 (5.8 –21.2)	19.6
Oral (LC_{50} ppm)				
Alder	360	0.49 ± 0.12	0.06 (0.001– 0.17)	27.7
Douglas-fir	360	0.58 ± 0.12	0.45 (0.16 – 1.13)	32.8

into the fourth instar. Ecdysis was disrupted in three principal ways, which were similar to those observed by Retnakaran et al. (1985): (1) ecdysis was prevented and the insect died within the old cuticle; (2) ecdysis was initiated, but was not completed and the larvae turned black and died; or (3) ecdysis was completed, but the old head capsule remained attached to the mandibular region of the new head capsule and larvae did not feed. Mortality of later instars and pupae was generally low if larvae successfully molted to fourth instars. We observed no external indications of cuticle deformation among surviving larvae, pupae, or adults.

Females reared on either diet and surviving topical treatment required significantly longer to pupate than did controls (Table 2). Development time of topically treated males reared on either diet was not affected by treatment. Females fed diflubenzuron in diet containing alder required significantly longer to develop than did untreated larvae (Table 3); development time of males was unaffected except at a dose of 0.5 ppm. On diet containing Douglas-fir, the larval development periods of control and treated females were not significantly different (Table 3). Males in this group did not show a significant effect overall, but took significantly longer than untreated larvae to pupate at doses of 0.25 and 0.5 ppm.

Pupal weights of larvae surviving treatment with diflubenzuron and reared either on alder or Douglas-fir foliage did not differ from those of controls, whether treatment was oral or topical (Table 4). Pupal weights of larvae reared on Douglas-fir were significantly lower than those of larvae fed alder but time to pupation was unaffected.

Foliar Chemistry. Total phenolic content was higher in alder than in Douglas-fir throughout the experimental period (mid-June through mid-August). Phenolic content of alder averaged 85.4 mg phenols/g dry tissue over this period (SD = 19.4; range, 55.6–106.0 mg phenols/g); Douglas-fir content averaged 36.4 mg/g (SD = 14.0; range, 20.3–66.4 mg/g).

Table 2. Larval development time (mean ± SD) of third-instar gypsy moth surviving topical treatment with diflubenzuron and fed alder or Douglas-fir foliage

Diet	Dose μg/g[a]	Days from treatment to pupation (n)[b] ♀♀		♂♂	
Alder					
	Control[c]	28.7 ± 2.7	(17)	24.4 ± 3.1	(27)
	0.25	29.5 ± 1.9	(13)	24.5 ± 1.9	(8)
	0.50	32.4 ± 2.9**	(9)	24.2 ± 1.7	(11)
	1.0	39.0 ± 4.8***	(4)	24.0	(1)
	2.0	39.0	(1)		(0)
	3.0		(0)	26.0	(1)
	All treated	32.2 ± 4.4**	(27)	24.4 ± 1.7	(21)
Douglas-fir					
	Control[c]	29.0 ± 1.7	(21)	24.7 ± 2.4	(24)
	0.2	30.1 ± 1.6	(8)	25.4 ± 1.1	(7)
	0.50	31.5 ± 4.1*	(6)	26.3 ± 3.4	(8)
	1.0	32.3 ± 2.9**	(4)	25.6 ± 2.3	(5)
	2.0	30.0	(1)	22.0 ± 2.4	(1)
	All treated	31.0 ± 2.8**	(19)	26.6 ± 2.4	(21)

*, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.001$, by t test.
[a] Larvae treated 48 h after molting to third instar.
[b] n, number of survivors.
[c] Larvae treated with acetone only.

Table 3. Larval development time (mean ± SD) of third-instar gypsy moth surviving oral treatment with diflubenzuron and fed alder or Douglas-fir foliage

Diet	Dose (ppm)[a]	Days from treatment to pupation (n)[b] ♀♀		♂♂	
Alder					
	Control[c]	29.6 ± 2.5	(20)	24.3 ± 2.3	(23)
	Control[d]	31.9 ± 3.3*	(19)	25.0 ± 2.5	(24)
	0.0625	30.4 ± 1.9	(10)	24.5 ± 1.3	(4)
	0.125	30.5 ± 1.3	(4)	24.0 ± 3.6	(6)
	0.25	33.2 ± 1.8**	(5)	26.3 ± 1.5	(4)
	0.50	29.0 ± 1.4	(2)	30.0 ± 4.2**	(2)
	1.0	32.8 ± 4.4*	(5)	27.0 ± 3.0	(3)
	2.0	30.5 ± 0.7	(2)	26.3 ± 1.5	(4)
	4.0	37.5 ± 2.1***	(2)	23.8 ± 3.8	(6)
	All treated	31.7 ± 3.0*	(30)	25.4 ± 3.1	(29)
Douglas-fir					
	Control[c]	31.3 ± 2.2	(24)	26.8 ± 2.0	(20)
	Control[d]	30.8 ± 2.8	(26)	26.8 ± 1.9	(16)
	0.0625	31.8 ± 2.9	(6)	27.5 ± 3.0	(12)
	0.125	32.4 ± 1.9	(5)	26.7 ± 2.1	(10)
	0.25	30.6 ± 3.2	(10)	28.6 ± 1.5*	(7)
	0.50	30.8 ± 1.1	(5)	29.0 ± 0.0*	(4)
	1.0	30.0 ± 1.4	(7)	27.0	(1)
	2.0	31.8 ± 1.3	(5)	25.8 ± 2.2	(5)
	4.0	32.6 ± 2.9	(5)	26.2 ± 1.8	(6)
	All treated	31.3 ± 2.8	(43)	27.2 ± 2.3	(45)

*, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.001$, by t test.
[a] Larvae treated 48 h after molting to third instar.
[b] n, number of survivors.
[c] Acetone added to diet.
[d] No acetone added to diet.

Table 4. Pupal weight (mean ± SD) of third-instar gypsy moth surviving treatment with diflubenzuron and fed alder or Douglas-fir foliage

Treatment/ diet[a]	Wt, g (n)[b]	
	♀♀	♂♂
Topical		
Alder		
Control[c]	1.50 ± 0.19 (17)	0.48 ± 0.08 (27)
All treated	1.43 ± 0.23 (27)	0.45 ± 0.08 (21)
Douglas-fir		
Control[c]	1.15 ± 0.14 (21)	0.44 ± 0.07 (24)
All treated	1.19 ± 0.15 (19)	0.46 ± 0.06 (21)
Oral		
Alder		
Control[d]	1.24 ± 0.20 (20)	0.46 ± 0.08 (23)
Control[e]	1.45 ± 0.20 (19)	0.44 ± 0.05 (24)
All treated	1.32 ± 0.31 (30)	0.42 ± 0.07 (29)
Douglas-fir		
Control[d]	0.94 ± 0.13 (24)	0.37 ± 0.05 (20)
Control[e]	0.93 ± 0.15 (26)	0.38 ± 0.06 (16)
All treated	0.91 ± 0.11 (43)	0.33 ± 0.05 (45)

[a] Larvae treated 48 h after molting to third instar.
[b] *n*, number of survivors.
[c] Larvae treated with acetone only.
[d] Acetone added to diet.
[e] No acetone added to diet.

We found no monoterpenes in alder. In Douglas-fir, total foliar monoterpene content averaged 4.9 mg/g dry weight during our study (SD = 1.1; range, 2.6–6.7 mg/g dry weight). The most abundant monoterpenes were alpha-pinene, beta-pinene, and sabinene, which made up 12.5, 35.1, and 31.6%, respectively, of the total monoterpenes from mid-June through mid-August.

The nitrogen content in alder leaves and Douglas-fir needles (previous year's growth) remained nearly constant during these experiments. The alder foliage fed to the larvae averaged 2.1% nitrogen over the experimental period (SD = 0.2; range, 1.8–2.4%); Douglas-fir foliage averaged 1.4% nitrogen (SD = 0.08; range, 1.2–1.5%). Nitrogen content was higher in alder than in fir at every sampling date.

Discussion

For a generalist herbivore such as gypsy moth, any association between larva and host plant is potentially dangerous: less suitable hosts may diminish or retard growth, lessen fitness, or adversely influence insecticide tolerance. On the other hand, characteristics of an "unsuitable" host, particularly its allelochemistry, may induce detoxifying enzymes, enhancing the ability of a larva both to change host plants if necessary (e.g., in times of heavy defoliation) and to tolerate insecticides. Furthermore, the ability of a larva to survive on a given host can be greatly enhanced or diminished by its parentage (Joseph et al. 1991).

In this study, larvae were more tolerant of diflubenzuron when they were reared on Douglas-fir than when they were reared on alder. These results are consistent with our analogous study involving carbaryl (Moldenke et al. 1992). Differential consumption of alder-based and Douglas-fir-based diet by larvae receiving diflubenzuron orally may have affected these results. However, most larvae on both diets had consumed most, but not all, of the treated diet after 48 h.

Sublethal effects were few. Development time of surviving females was lengthened by treatment, with the exception of those reared on Douglas-fir and treated orally. In the field, prolonging the developmental period might affect the nutritional suitability of the host plant, the susceptibility of the larva to predators and parasitoids, or the availability of adult males for mating.

In the field, diflubenzuron is applied as a spray. Thus, insects eating foliage are exposed to diflubenzuron both topically and orally. Although diflubenzuron is considered to be generally, though not always, ineffective when applied topically (Maas et al. 1980, Mauchamp & Perrineau 1987, Retnakaran & Wright 1987), we found the LD_{50} for topical application to be lower than that of topically applied carbaryl (Moldenke et al. 1992). Abdelmonem & Mumma (1981), who incorporated diflubenzuron in artificial diet fed to third instars of gypsy moth, reported an LC_{50} for failure to molt to the fourth instar (0.052 ppm) similar to the LC_{50} we obtained for larvae feeding on alder. Granett & Dunbar (1975), however, reported a considerably lower LC_{50} (0.013 ppm) for third-instar gypsy moth receiving the compound in artificial diet, and Granett & Weseloh (1975) reported a still lower EC_{50} (0.0075 ppm).

The effective toxicity of diflubenzuron and related compounds seems to be controlled by the rate at which they are metabolized (Maas et al. 1980, Neumann & Guyer 1987). The extent and type of metabolism involved are not resolved, however, and probably vary among species (Maas et al. 1980, Retnakaran et al. 1985, Nakagawa et al. 1989). Overall, the primary role in detoxication of diflubenzuron has been ascribed to hydroxylation or oxidation by polysubstrate monooxygenases (PSMOs) (Chang 1978, Ivie & Wright 1978, Pimprikar & Georghiou 1979), hydrolysis by esterases and diflubenzuron hydrolases (Metcalf et al. 1975, Ishaaya & Degheele 1988, Gazit et al. 1989, Van Laecke & Degheele 1991), and conjugation by glutathione transferase (Chang 1978, Ivie & Wright 1978, Gazit et al. 1989, Van Laecke & Degheele 1991).

Many studies have reported correlations among ingestion of allelochemicals, induced activities of detoxifying enzymes (particularly PSMOs), and increased tolerance of insects to insecticides (Terriere 1984, Brattsten 1988). Studies of induction in gypsy moth (Ahmad &

Forgash 1978; Sheppard & Friedman 1989; Lindroth et al. 1990, 1991) have provided mixed results. The several enzyme systems involved appear to differ substantially in their response to both nutrient levels and allelochemicals; even within enzyme systems, results are not consistent. On the basis of our previous findings (Moldenke et al. 1992) and of enzyme data collected contemporaneously with this diflubenzuron study (A.F.M., unpublished data), we ascribe the higher tolerance of Douglas-fir-fed larvae to probable induction of detoxifying enzymes by the terpenes in the Douglas-fir. Induction also may have occurred in response to the low nitrogen levels, the specific phenolics, or both in Douglas-fir, which may have increased esterase activity in particular (Lindroth et al. 1990, 1991).

Acknowledgments

We thank P. Barbosa and P. Gross (Department of Entomology, University of Maryland, College Park) for furnishing the egg masses. G. Joseph, A. Berry, and M. Wernz (Oregon State University) provided unstinting technical support. This research was supported by USDA–CRGO Pest Science Grant 87-CRCR-1-2478. This is Technical Paper No. 9941 of the Agricultural Experiment Station, Oregon State University.

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Received for publication 30 July 1992; accepted 16 November 1992.