Effect of Topsoil Treated with J2581 on Reproduction in the Oriental Fruit Fly (Diptera: Tephritidae)

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ABSTRACT Prepupae of the oriental fruit fly, *Dacus dorsalis* Hendel, were exposed to topsoil treated with a benzyl-1,3-benzodioxole derivative (BBD), J2581 (5-ethoxy-6-[4-methoxyphenyl]methyl-1,3-benzodioxole). At a concentration range of 0.83–13.20 mg J2581 per g of soil, pupation, adult emergence, and egg production were not affected, but egg hatch was significantly reduced. J2581 activity in soil was retained for ≥30 d at concentrations >4 mg/g soil. When soil was subjected to temperatures of 25, 35, and 45°C for 1 h, activity was reduced. The activity was still significantly higher than in soil treated with acetone alone. Crossing experiments between treated and control males and females revealed that males were primarily affected by J2581. Spermatozoa in the testes of treated males lacked motility. Spermatozoa were also absent in a significant number of spermathecae from females mated to treated males. Rec-assays for potential mutagenicity of 10 BBDs were all negative.

KEY WORDS Insecta, fruit flies, benzodioxole, soil

TOPICAL OR ORAL administration of several methylenedioxy derivatives of substituted benzylphenols (benzyl-1,3-benzodioxoles) (BBDs) in insects causes temporary or permanent interference with reproduction (Jurd et al. 1979, Rawlins et al. 1979, Chang et al. 1980, Flint et al. 1980, Nagasawa et al. 1980, Langley et al. 1982, Van Mellaert et al. 1983, Chang et al. 1984, Nelson et al. 1985, Song 1988). Other effects of BBDs and related benzylphenols include interference with sex attractancy (Chang et al. 1984) and induction of precocious metamorphosis (Darvas et al. 1988).

We tested BBDs on larvae and adults of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) and found that several interfere with reproduction (Chang et al. 1984, 1988; Hsu et al. 1989). In the study reported here, we assessed the effects of J2581 (5-ethoxy-6-[4-methoxyphenyl]methyl-1,3-benzodioxole) in soil on reproduction of oriental fruit fly, *Dacus dorsalis* Hendel. Eight BBDs used in our previous studies were nonmutagenic in the Ames/*Salmonella* microsome assay (Ames et al. 1975) with and without metabolic activators (Hsu et al. 1989). We retested these BBDs and two others in the more sensitive Rec-assay (Kada 1980) for potential mutagenicity.

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Materials and Methods

Insects. Third (last) instars of *D. dorsalis*, as well as *C. capitata* and the melon fly, *Dacus cucurbitae* Coquillett, were obtained from the mass-rearing facilities of the USDA–ARS Tropical Fruit and Vegetable Research Laboratory in Honolulu. Larvae and adults were fed on a wheat and a sugar-yeast hydrolysate diet, respectively (Tanaka et al. 1969).

Chemicals. All BBDs were synthesized by one of us (L.J.) and their structures confirmed by elemental (C,H), proton nuclear magnetic resonance, and mass spectral analysis (Jurd et al. 1979). J2581 (5-ethoxy-6-(4-methoxyphenyl) methyl-1,3-benzodioxole) was used for Experiments 1–4; J2581, J2710 (5 - methoxy - 6 - (1 - [4 - methoxyphenyl] ethyl) - 1, 3-benzodioxole), J2922 (5-ethoxy-6-(1-[4-methoxyphenyl]ethyl)-1,3-benzodioxole), J2962 (5-ethoxy-6-(phenylethyl)-1,3-benzodioxole), J3223 (5-ethoxy-6-(1-[4-ethoxyphenyl]ethyl)-1,3-benzodioxole, J3263 (5-methoxy-6-(1-[4-ethoxyphenyl]ethyl)-1,3-benzodioxole), J3360 (5-methoxy-6-(1-[4-fluorophenyl]ethyl)-1,3-benzodioxole), J2335 (5-methoxy-6-(4-methoxyphenyl)methyl-1,3-benzodioxole), J3230 (5-(2-propenyloxy)-6-[1-(4-methoxyphenyl] ethyl]-1,3-benzodioxole), and J3371 (5-ethoxy-6-(1-[4-ethoxyphenyl] isopropyl-1,3-benzodioxole) were used for experiment 4. A purity of >99% for all BBDs was determined by development of silica gel sheets (Eastman Kodak, Rochester, N.Y.) in ethyl acetate/hexane (3:7, vol/vol) and densitometric scanning of the acid charred sheets. High-pressure

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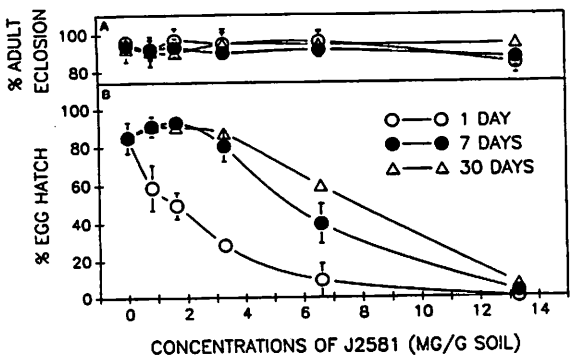

Fig. 1. Percentage (A) eclosion of adults of the oriental fruit fly exposed as prepupae to soil treated with various concentrations of J2581. Data points at 0 concentration of J2581 represent acetone controls and (B) hatch from eggs oviposited by oriental fruit flies exposed as prepupae to soil treated as above, but aged for 1, 7, or 30 d. Error bars, SEM.

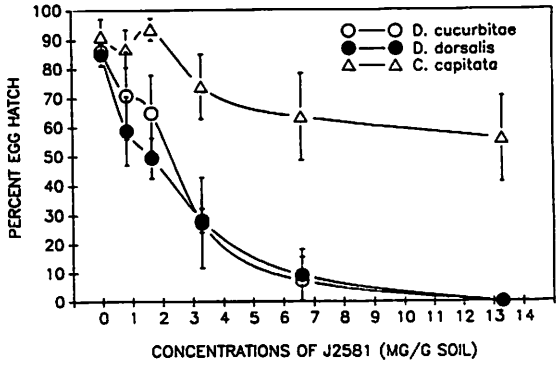

Fig. 2. Percentage hatch from eggs oviposited by the oriental and Mediterranean fruit flies and melon fly exposed as prepupae to soil treated with various concentrations of J2581. Data points at 0 concentration of J2581 represent acetone controls. Error bars, SEM.

liquid chromatography (HPLC)-grade acetone was used as the diluent in preparing various concentrations of J2581 before treatment of soil.

Soil Treatment. Dry, nonsterilized top soil (pH 6.2, 0.15% total nitrogen, 0.93% organic carbon, salinity 6.4 millimohs/cm) was sifted through a 1-mm mesh screen. Twenty ml of acetone containing J2581 was mixed with 30 g of soil so that concentrations of 0.83, 1.66, 3.30, 6.60, and 13.20 mg J2581 per g of soil were obtained after drying in air.

Treatment of Insects. As in previous experiments (Chang et al. 1988, Hsu et al. 1989), four criteria were used to assess the effect of a BBD on flies. These were pupation, adult emergence, egg production, and egg hatch.

At least four replications of 100 late instars were placed in 10-g wheat diet in a 3-cm-diameter glass dish. The dish was placed into a larger container packed with 30 g of soil treated with a given concentration of J2581 or acetone (control). The container was covered with organdy cloth to prevent escape of larvae. Prepupae displayed springing behavior that allowed them to jump from the rearing medium onto the soil where they pupariated in ≈11–12 h. After 7 d in soil, pupae were placed in cages. Upon eclosion, 50 males and females were placed in mating/oviposition containers (Chang et al. 1988). For some experiments, the sex of flies was determined immediately after eclosion. Flies were then held in separate mating/oviposition containers before mating with control flies (see experiment 3 below).

The number of eggs oviposited per treatment group was recorded over a 30-d period. Of the eggs oviposited daily, ≥4 groups of 100 eggs were removed and egg hatch determined over 40 d. All experiments were conducted at 23°C.

Experiment 1: Retention of J2581 Activity in Soil Versus Time. To test how long soil treated with J2581 retained its activity, four groups of 100 prepupae were exposed for 7 d to soil treated within 24 h with J2581, soil treated 7 d earlier with J2581, and soil treated 30 d earlier with J2581. A limited experiment was also conducted with J3223 to compare its activity with that of J2581. Percentage pupation, adult emergence, egg production, and egg hatch were determined for treated and control groups.

Experiment 2: Retention of J2581 Activity in Soil Versus Temperature. Soil treated with J2581 (13.2 mg/g soil) was subjected for 1 h to temperatures of 25, 35, or 45°C in a dry oven. Four groups of 100 prepupae were then exposed to treated soil from each temperature regime. Percentage pupation, adult emergence, egg production, and egg hatch were determined for treated and control groups.

Experiment 3: Effect of J2581 on Males and Females. To test whether males or females were most affected by treated soil, four groups of 50 treated male or female flies were allowed to mate with an equal number from a control group. Controls consisted of flies of both sexes exposed as prepupae to soil treated with acetone only. From these

Table 1. Percentage egg hatch after exposure of *D. dorsalis* prepupae to J3223 in topsoil (100 ppm)

Age of flies, d	Percentage egg hatch ($\bar{x}$ ± SEM)		$P > \chi^2$ (df = 1)
	J3223	Acetone	
10	0	50.4 ± 5.8	0.002
15	0	83.6 ± 2.2	0.004
20	1.6 ± 3.0	87.6 ± 7.1	0.008
25	30.8 ± 7.3	73.6 ± 3.8	0.009
30	19.2 ± 7.6	77.2 ± 5.9	0.009
35	7.2 ± 3.3	68.0 ± 7.6	0.009

Percentage egg hatch calculated from five replicates of fifty eggs each randomly taken from a group oviposited by flies of a specified age.

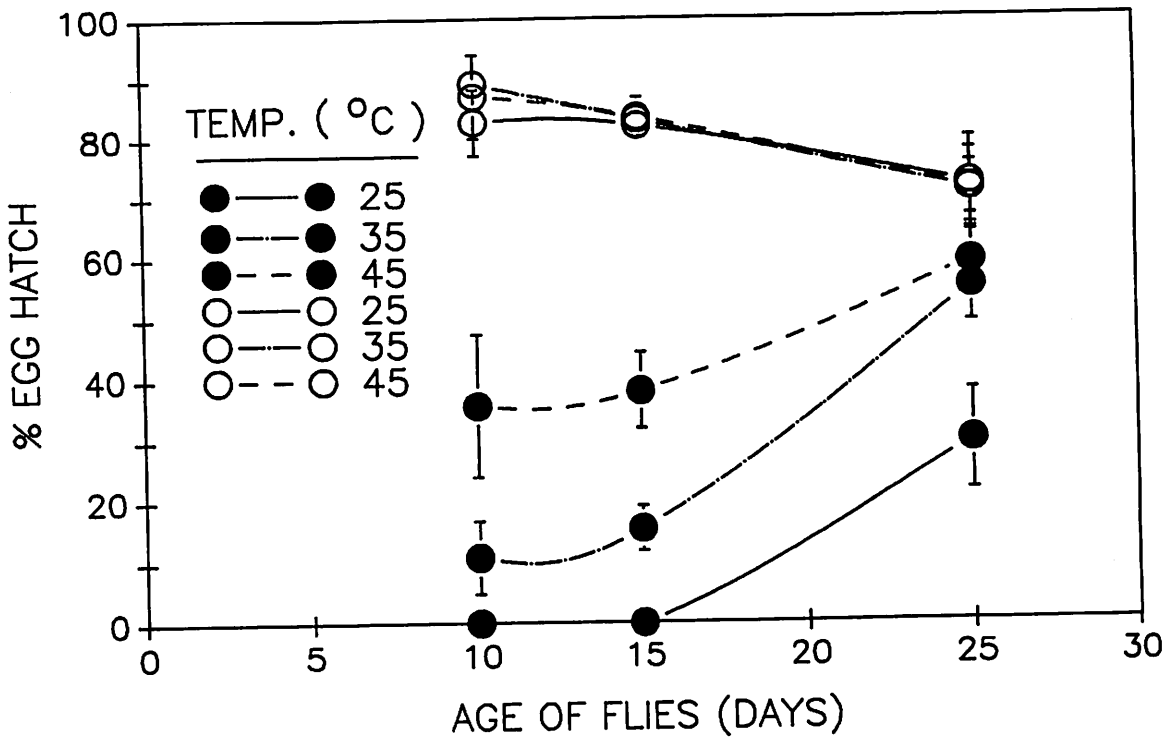

Fig. 3. Percentage hatch from eggs oviposited by 10-, 15-, and 25-d-old oriental fruit flies exposed as prepupae to soil treated with J2581 (13.20 mg/g soil) and subjected for 1 h to 25, 35, and 45°C. Solid circles = J2581-treated; open circles = acetone-treated. Error bars, SEM.

groups, testes and ovaries from 10-d-old flies were removed and each examined for abnormalities.

Experiment 4: Rec-Assay of 10 BBDs. The Rec-assay is based on the sensitivity of recombinant repair deficient M45 rec-strains of *Bacillus subtilis* Cohn to potential mutagenic agents. The H17 rec$^+$ strain was used as a control. Broth agar plates were streaked with either strain H17 rec$^+$ or M45 rec$^-$. Eight-millimeter paper disks were placed over the starting points of the streaks and 10 μl each of a BBD (2 mg/ml dimethyl sulfoxide) were added to each disk. The plates were incubated overnight at 4°C and then at 37°C for 20 h, after which the lengths of the inhibition zones were measured. Sodium azide (1.75 *M*) and sterile water were used as the positive and negative controls respectively. Dimethyl sulfoxide (DMSO) served as the solvent control.

Data Analysis. Comparisons of the numbers of larvae pupated, adult emergence, eggs oviposited, and egg hatch between treated and control groups were made by analysis of variance and the Waller-Duncan *k* ratio procedure ($P < 0.01$; PROC GLM; SAS Institute 1982) except in the case of J3223 where the Kruskal-Wallis Test ($P < 0.01$; PROC NPARIWAY; SAS Institute 1982) was used. Significance of differences in inhibition zone measurements in the Rec-assay were analyzed by *t* test ($P = 0.01$).

Results

Experiment 1: Retention of J2581 Activity in Soil Versus Time. Concentrations of J2581 from 0.83 to 13.20 mg/g soil did not affect pupation (data not shown), adult emergence (Fig. 1A), or egg production (data not shown) of the oriental fruit fly. Concentrations of J2581 from 0.83 to 13.20 mg/g soil resulted in a decrease in egg hatch (Fig. 1B). These results were also obtained whether pupae were removed 1 d or 7 d after exposure to soil containing J2581 (data not shown). Fig. 1B also shows that soil retained J2581 activity for ≥30 d at concentrations >4 mg/g soil. At lower concentrations of J2581 (0.83–3.30 mg/g soil), soil treated 1 d previously with J2581 showed the highest retention of activity ($F = 80.30$; df = 18; $P \leq 0.0001$).

In other related experiments, the effect of J2581 on *D. dorsalis* and *D. cucurbitae* over the 0.83–13.20 mg/g soil concentration range was identical in respect to percentage of egg hatch; however, the effect of J2581 on *C. capitata* was significantly less than on the other two tephritid species (Fig. 2). A significant decrease in egg hatch was still evident in eggs oviposited by 35-d-old flies treated as prepupae with another BBD, J3223, at a concentration of 0.1 mg/g soil (Table 1).

Experiment 2: Retention of J2581 Activity in Soil Versus Temperature. Fig. 3 shows that soil

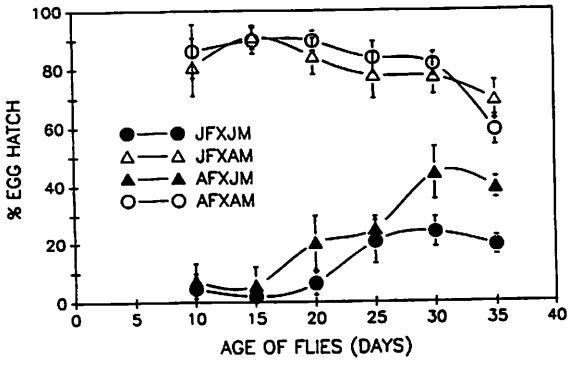

Fig. 4. Percentage hatch from eggs oviposited from 10 to 35 d after adult eclosion by oriental fruit flies exposed as prepupae to soil treated with J2581 (6.60 mg/g soil). Treated female (JF) and male (JM) flies were mated with each other as well as with flies exposed to soil treated with acetone alone (AF, AM). Error bars, SEM.

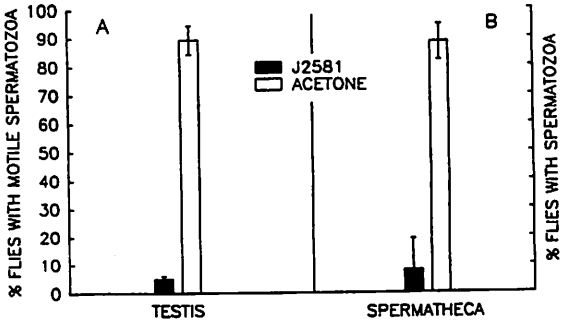

Fig. 5. Percentage (A) of oriental fruit fly males with motile spermatozoa in testes. Males exposed as prepupae to soil treated with J2581 (6.60 mg/g soil) and (B) of oriental fruit fly females with spermatozoa in spermathecae. Females exposed as prepupae to soil treated with J2581 (6.60 mg/ml) and mated with males treated with J2581 or acetone.

treated with J2581 and exposed for 1 h to temperatures from 25 to 45°C significantly retained activity compared with soil treated with acetone alone ($F = 224.67$; df = 30; $P < 0.0001$). However, comparisons between soils exposed to heat showed decreased activity for each 10°C rise in temperature.

Experiment 3: Effect of J2581 on Males and Females. Fig. 4 shows that egg hatch was significantly decreased when one of the mating pairs was a male treated with J2581 ($F = 384.41$; df = 20; $P \leq 0.0001$). Mating pairs of females treated with J2581 and control males did not result in a significant decrease in egg hatch compared with controls ($F = 384.41$; df = 20; $P \leq 0.0001$). Ovaries and testes removed from mating pairs from each test group showed no external abnormalities. However, spermatozoa from males treated with J2581 were less motile than spermatozoa of controls (Fig. 5A). Spermatozoa were absent in spermathecae of a significant number of flies mated to males treated with J2581 (Fig. 5B). Spermatozoa were present in spermathecae of mated flies from test groups in which males treated with J2581 were excluded.

Experiment 4: Rec-Assay of 10 BBDs. Of 10 BBDs tested in the Rec-assay, only J3360 and J2962 showed zones of inhibition which could be measured. However, both BBDs did not significantly revert rec⁻ to rec⁻ when differences in inhibition lengths (mm) were compared with sodium azide used as the positive control (J3360: $t = -13.43$, $P = 0.0002$; J2962: $t = -13.53$, $P = 0.0002$) (Table 2). Both water and DMSO solvent controls were negative in the assay.

Discussion

To our knowledge, this is the first study of the effects on insects of a BBD incorporated in soil. The oriental fruit fly, like other tephritids, pupates in the soil. A significant reduction in egg hatch resulted from exposure of oriental fruit fly prepupae to topsoil treated with J2581. Similar results were also found with two other tephritid flies, *D. cucurbitae* and *C. capitata*. Within the range of J2581 concentrations used in this study (0.83–13.20 mg/g soil), effects on pupation, adult emergence, or egg production were not apparent. The soil could retain activity for ≥30 d in concentrations of J2581 >4 mg/g soil. Even though activity was gradually lost when soil was heated from 25 to 45°C, it was still significantly higher than in the soil treated with acetone alone.

Clearly, the high concentrations of J2581 used for soil treatment would preclude their practical use. However, the benzyl-1,3-benzodioxoles and related benzylphenols are amenable to simple molecular modifications to increase their activity (Ma-

Table 2. Results of rec-assay on benzyl-1,3-benzodioxole derivatives with *B. subtilis* strains H17 and M45

Test compound	Inhibition length, mm			Rec effect
	H17 (Rec+)	M45 (Rec−)	Difference	
J2335	0	0	0	−
J2581	0	0	0	−
J2710	0	0	0	−
J2922	0	0	0	−
J2962	0	0.83	0.83	−
J3223	0	0	0	−
J3230	0	0	0	−
J3263	0	0	0	−
J3360	0	0.67	0.67	−
J3371	0	0	0	−
Na azide	0	22.50	22.50	+
Water	0	0	0	−
DMSO	0	0	0	−

Benzodioxole compounds were dissolved in DMSO (2 mg/ml). Each disk contained 20 μg BBD. Sodium azide (1.75 *M*) and sterile water served as the positive and negative controls, respectively. DMSO served as the solvent control. Data are the mean of three replications. SEM for J2962, J3360, and sodium azide were ±0.23, ±0.23, and ±2.50, respectively.

tolcsy et al. 1986). For example, one BBD which has shown much promise is J3223, which differs from J2581 by having an ethoxy instead of methoxy group on C-4 of the phenyl ring. This BBD has shown significant activity in soil against the oriental fruit fly at a concentration as low as 100 ppm.

Matings between males and females exposed as prepupae to soil treated with J2581 showed that males were principally affected. A significant reduction in egg hatch resulted only if mating groups included treated males.

Testes and ovaries from treated flies had no external abnormalities. However, mature spermatozoa in testes from treated males were not motile. In comparison, spermatozoa from control flies were fully active. Also, an absence of spermatozoa was observed in spermathecae from a significant number of flies mated with males treated with J2581. Apparently, males treated with J2581 either were unable to transfer spermatozoa to females during mating, or spermatozoa were not able to migrate to the spermathecae. Such effects could explain the significant reduction in egg hatch that resulted from exposure of oriental fruit fly prepupae to soil treated with J2581. Chang et al. (1980) also found that in the house fly (*Musca domestica* L.), J2922 reduced the ability of male flies to transfer spermatozoa. However, our finding that J2581 affects the motility of spermatozoa in oriental fruit flies and, in effect, sterilizes males, is in variance with the conclusion of Chang et al. (1980) that at the nontoxic doses tested, J2922 was inactive as a sterilant in male house flies. We have not determined whether this discrepancy is due to differences in the insects or in BBDs used.

Greater susceptibility of males than females to J2581 when the BBD was administered during the immature stage is further supported by feeding experiments. In these experiments, flies reared as larvae on a diet containing J2581 or acetone only (controls) were allowed to mate. A significant reduction in egg hatch resulted only if one of the mating pair was a treated male (findings to be published elsewhere). We also found that topical or oral administration of BBDs to newly emerged *C. capitata* adults primarily affected females (Chang et al. 1988, Hsu et al. 1989). Evidently, males in the immature stage are more susceptible to BBDs than females; the opposite occurs in the adult stage. A partial explanation may lie in the earlier development of spermatozoa compared with oocytes in *D. dorsalis* and *C. capitata*. Mature spermatozoa are already present in newly emerged males, while mature oocytes do not develop until 2 d or more after eclosion. Spermatogenesis in flies may be particularly vulnerable to BBDs, particularly in the early stage of adult development. Likewise, oogenesis may be particularly sensitive to BBDs in the short period immediately following adult eclosion (Hsu et al. 1989).

Multiple biological functions have been attributed to several derivatives of benzyl-1,3-benzodioxoles and related benzylphenols. These include inhibition of juvenile hormone synthesis (Brookes et al. 1985), precocious development (Darvas et al. 1988), vitellogenesis (Van Mellaert et al. 1983, Chang et al. 1988, Song 1988), sex attractancy (Chang & Hsu 1982), cytochrome P450 monooxygenase activity (Matolcsy et al. 1986), tubulin polymerization and mitosis (Batra et al. 1984), and insect reproduction in general (Rawlins et al. 1979, Flint et al. 1980, Langley et al. 1982, Nelson et al. 1985).

Hsu et al. (1989) evaluated eight BBDs mentioned in the research literature for potential mutagenicity in the Ames/*Salmonella* microsome test with and without metabolic activators and found all to be negative. We further retested these eight BBDs and two others in the more sensitive Rec-assay (Kada et al. 1980) and again found all to be negative. *B. subtilis* is useful in the Rec-assay because its cellular membrane is more permeable to chemicals than that of some gram-negative enteric bacteria used in the Ames reversion assays (Kada et al. 1980).

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