Insecticidal Activity of Floral, Foliar, and Root Extracts of *Tagetes minuta* (Asterales: Asteraceae) Against Adult Mexican Bean Weevils (Coleoptera: Bruchidae)

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ABSTRACT Experiments were conducted to determine speed of action and toxicities of extracts of *Tagetes minuta* L., a source of naturally occurring insecticidal compounds. LC_{50} values for male and female Mexican bean weevils, *Zabrotes subfasciatus* (Boheman), were determined for floral, foliar, and root extracts of *T. minuta*. The 24-h LC_{50} values ranged from 138 $\mu g/cm^2$ for males exposed to the root extract (most susceptible) to 803 $\mu g/cm^2$ for females exposed to the foliar extract (least susceptible). Increasing the duration of exposure to 48 h decreased all LC_{50} values 20–30 $\mu g/cm^2$. Males were more susceptible than females. The time to incapacitation for 50% of the test insects (IT_{50}) for floral and foliar extracts indicated fast-acting, volatile components, whereas the root extract data indicated slower-acting components, likely a result of the interaction of photophase with time-dependent efficacy. Floral and foliar extracts of *T. minuta* may be useful as insecticides for controlling stored-product pests.

KEY WORDS *Zabrotes subfasciatus, Tagetes minuta,* extracts

MARIGOLDS, *Tagetes* spp., are a useful intercrop in agriculture. Populations of *Meloidogyne* spp. nematodes have been reduced by intercropping marigolds (Davide 1979, Huang 1984). Other cultural applications of *Tagetes* spp. include the use of *T. minuta* L. leaves to repel mosquitos and safari ants (Maradufu et al. 1978) in Kenya.

Researchers have isolated several insecticidal compounds from *Tagetes* spp. Compounds extracted from the leaves and flowers of *T. minuta* are toxic to *Aedes aegypti* (L.) larvae (Maradufu et al. 1978). Laboratory tests using polyacetylenes from the Asteraceae indicated that α-terthienyl, a phototoxic thiophene from *Tagetes* spp., had an LC_{50} of 19 ppb for *A. aegypti* larvae when combined with near-UV radiation (Arnason et al. 1981). Morallo-Rejesus & Decena (1982) isolated α-terthienyl and 5-(3-buten-1-ynyl)-2,2-bithienyl as the active insecticidal components from root extracts of *T. erecta* L. The topical LD_{50} of partially purified *T. erecta* root extract was 8.1 mg/g for *Rhyzopertha dominica* (F.) and 4.3 mg/g for *Tribolium castaneum* (Herbst) (Morallo-Rejesus & Decena 1982). Numerous studies have evaluated the insecticidal properties of α-terthienyl and its analogs (Philogene et al. 1985, 1986; Arnason et al. 1986, 1988, 1989; Champagne et al. 1986; Evans et al. 1986; Hasspieler et al. 1988, 1990; Sen et al. 1990).

The toxicokinetics of α-terthienyl were also examined for three species of Lepidoptera (Iyengar et al. 1987). The topical LD_{50} for *Manduca sexta* (L.) was 10 μg/g, but for *Heliothis virescens* (F.) it was 470 μg/g and for *Ostrinia nubilalis* (Hübner) the LD_{50} was 700 μg/g (Iyengar et al. 1987). This difference was likely caused by a more rapid clearance of the toxin by the more tolerant species, which may have preadapted rapid elimination of this toxin via evolutionary associations with the Asteraceae (Iyengar et al. 1987). This elimination was facilitated by higher levels of cytochrome P450 in those species (Iyengar et al. 1990).

Generally, the efficacy of α-terthienyl against various mosquito larvae may also be related to the limited evolutionary association between the compound and these insects, with the oxidative mode of action (Hasspieler et al. 1990) being enhanced by the lack of facile or rapid detoxification. This suggests that other naive insects, such as stored-product pests, may be susceptible

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to these compounds. Many stored-product pests were (and probably still are) either fossorial, litter-decomposing species or specialists on the seeds of plants in the Leguminoseae and Graminaceae families before synanthropic association. Thus, they could be susceptible to compounds evolved against herbivory of tissues of other plant groups. Our objective was to evaluate tissue extracts of *T. minuta* against Mexican bean weevils, *Zabrotes subfasciatus* (Boheman), a sexually dimorphic bruchid of economic importance throughout the world. We are also concerned with the production of "insecticidal crops" by developing countries as an alternative to synthetic insecticides. *Tagetes* spp. have been proposed as an insecticidal crop to provide a locally available source of α-terthienyl for mosquito control in developing countries (Arnason et al. 1981). However, in practice, potential insecticidal crops, such as *T. minuta,* should be fully utilized, rather than to simply serve as a source of a single chemical component. The efficacy of extracted material from all tissues should be determined so that the entire plant can be used, if possible.

Materials And Methods

Plant Culture. Seeds of Mexican marigold, *T. minuta* 'Muster John Henry', were planted in shallow trays (23 by 45 cm) containing sterile soil (Pro-Mix, Park Seed, Greenwood, SC) and placed in a naturally illuminated ventilated greenhouse maintained at 27°C and 80% RH. After germination, the seedlings were transplanted into "Peat Pots" (Park Seed, Greenwood, SC) and grown in the greenhouse until they were ≈6 wk old. The plants were fertilized at biweekly intervals using a dilute fertilizer solution (Peters 20:20:20 General Purpose Fertilizer). Greenhouse insect pests were controlled using preformulated (2% [AI] vol:vol) insecticidal soap (Safers Insecticidal Soap, Park Seed, Greenwood, SC) applied to run off. Six-week-old plants were transplanted to 10 rototilled 10-m rows in a field plot (10 by 15 m) at the University of Alabama Arboretum in Tuscaloosa, AL. A granular fertilizer (Fertilome 13:13:13) was applied to the plot before transplanting. Supplementary water was added by porous soaker hoses and sprinklers. The seedlings were transplanted ≈0.3 m apart. Plants were pruned as necessary to induce branching from the main stalk and to remove wilted or dead material.

Preparation of Extracts. Root and foliage material was harvested over a 2 to 3-wk period ≈2 mo before maximum bloom. The root material was washed with tap water at harvest. All plant portions were placed in large plastic self-sealing bags, sealed, and transported to the laboratory, where the bags were opened, flushed with nitrogen, and resealed. The material was stored in a 10°C refrigerator until extraction. Two hundred grams of plant material were placed in a 3,000-ml round-bottom three-neck flask and covered with distilled water. This flask was connected to vapor-arm A of the Lickens & Nickerson (1964) distillation extractor (Kontes Scientific Glassware and Instrumentation, Vineland, NJ) (Fig. 1). A 100-ml round-bottom boiling flask containing 25 ml of HPLC-grade methylene chloride was connected to vapor-arm B. The extractor was fitted with a Liebig condenser (Kontes, Vineland, NJ) through which cool water was circulated through the condenser to reduce the loss of volatile components. The extraction was carried out for 5 h according to the procedure of Godefroot et al. (1981). The resulting extracts were concentrated with a stream of purified nitrogen while gently heating the flask with a heating mantle. The concentrated extracts were deposited in 7-ml glass vials equipped with a Teflon-lined screw-cap under a nitrogen headspace and stored at −20°C before analysis. Samples for in-

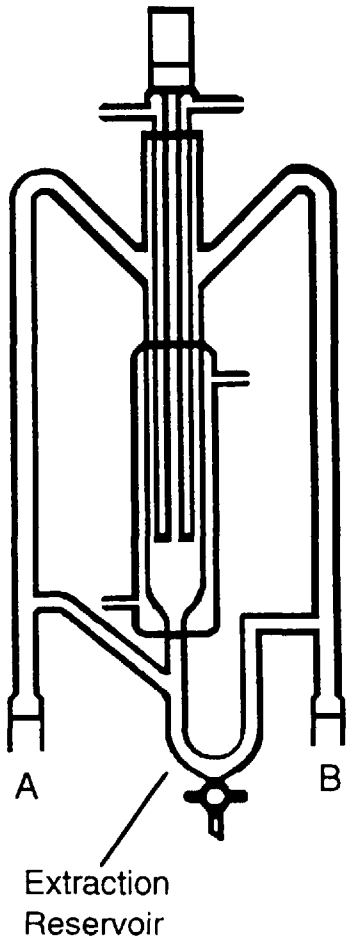

Fig. 1. Schematic diagram of the preparative-scale simultaneous steam distillation and extraction apparatus.

sect bioassay were express-shipped to Montana State University and stored at −20°C.

Chromatography and Identification of Chemical Components. Gas chromatographic profiling was performed on a chromatograph (Perkin-Elmer Sigma One) equipped with a split/splitless injector and a flame-ionization detector. An integrator (Hewlett-Packard 3393A) connected to a disk storage system (Hewlett Packard 9114A) was used to store and replot the data. A fused silica capillary column (25 m by 0.25 mm i.d.) coated with a 0.33-μm film of cross-linked 5% phenyl methyl silicone (HP-5) was used with helium as the carrier gas. A precolumn (60 cm by 0.32 mm i.d.) coated with the same stationary phase and film thickness was connected to the column using a universal press-fit connector to reduce potential deterioration of the column caused by injection of nonvolatile material. The column oven was held for 1 min at 50°C during a 1-min splitless injection, then programmed at 3°C/min to 300°C and held for 20 min. Injector and detector temperatures were 220 and 250°C, respectively. Identification of volatiles was achieved using a mass selective detector (Hewlett-Packard 5970) coupled to a gas chromatography system (Hewlett-Packard 5890) under chromatographic conditions as described above. Electron impact spectra were obtained at 70 eV by scanning from 40 to 300 a.m.u. at ≈1.65 scans/s. Structural elucidation was achieved by searching a database of essential oil spectra using a probability based matching algorithm and comparing with spectra reported in the literature.

Insect Culture. *Z. subfasciatus* were reared on a diet of dried Pinto beans (*Phaseolus vulgaris* L.) with an equilibrium moisture content of 13.7 ± 0.9% (oven dry method). Culture conditions were 27 ± 1°C, 65 ± 5% RH, and a photoperiod of 12:12 (L:D) h. Cultures were maintained in 0.95-liter glass jars containing 0.5 liter of equilibrated beans. Beans were inoculated with ≈300 bruchid adults, and culture diet was discarded after emergence of two successive generations.

Dose-Response Assay. *T. minuta* foliar and floral extracts were diluted in absolute ethanol (Quantum Chemical, St. Louis, MO) to concentrations of 100, 500, and 1,000 μg/cm^2 on filter paper (Whatman No. 1) when applied in a 0.5-ml aliquot. The *T. minuta* root extract was similarly diluted to concentrations of 100, 250, 400, and 500 μg/cm^2. Aliquots were applied to 5.5-cm filter paper disks in the inverted lids of glass petri dishes 5.0 cm in diameter. The ethanol was evaporated for 20 min before adding five male and five female Z. *subfasciatus* (0–24 h after adult emergence). The insects were immediately covered with the inverted bottom of the petri dish. To avoid disturbing volatile equilibria early in the bioassay, knockdown was assessed visually at 0.25, 3, 6, and 12 h for the foliar and floral extracts and at 0.25, 6, and 18 h for the root extract. At 24 and 48 h, mortality and moribundity were determined for the foliar and floral extracts; the root extract was evaluated at 24 h only. Moribundity was determined by righting an insect that was weakly attempting to ambulate on its back; insects that failed to remain upright were considered moribund. Mortality was determined by probing insects three times with a blunt dissecting probe; insects that failed to move were considered dead. All moribund insects subsequently died, thus data from them were pooled with the mortality data for analyses. Ten replicates of each concentration were prepared. All trials were conducted at 65 ± 8% RH, 27 ± 2°C, and a photoperiod of 12:12 (L:D) h and commenced at a similar time in the photophase of the diel cycle.

Statistical Analyses. Regression lines for log concentration (μg/cm^2) at 24 and 48 h were estimated by probit analyses based on corrected data (SAS Institute 1988). The temporal counts on incapacitated insects follow a serial time-dosage-mortality pattern that is not suitable for probit analysis because of correlation between counts at successive time intervals. However, the data can be analyzed as a percentage of incapacitated insects per number of unaffected insects at the beginning of each time interval (Robertson & Preisler 1992). The effects of sample times are treated as categorical variables that are added to the effect of concentration and analyzed using a complementary log-log model with a binomial error distribution (Preisler & Robertson 1989). Controls of concentration 0 are included by displacing all concentrations with a small positive amount (Tukey et al. 1985). Maximum likelihood estimates of conditional mortality probabilities and the effect of concentration are obtained from the fitted model, which are used to calculate probabilities of mortality for each exposure period and concentration (Robertson & Preisler 1992). Estimates of the speed to incapacitation of half of the susceptible insects (SIT_{50}) and half of all trial insects (IT_{50}), including those not susceptible during the bioasssay, can be determined by mathematical interpolation (Preisler & Robertson 1989). Models of parallelism and equality (for extracts or sexes) of the linear predictor of the model can be tested (Robertson & Preisler 1992). Our analyses were conducted using GLIM (Payne 1987) and programmed according to Robertson & Preisler (1992).

Results

The chemical composition of the extracts from the flowers, leaves, and roots varied qualitatively and quantitatively (Fig. 2; Table 1). The root extract (Fig. 2C) contained more thiophenes (compounds identified with retention times >30

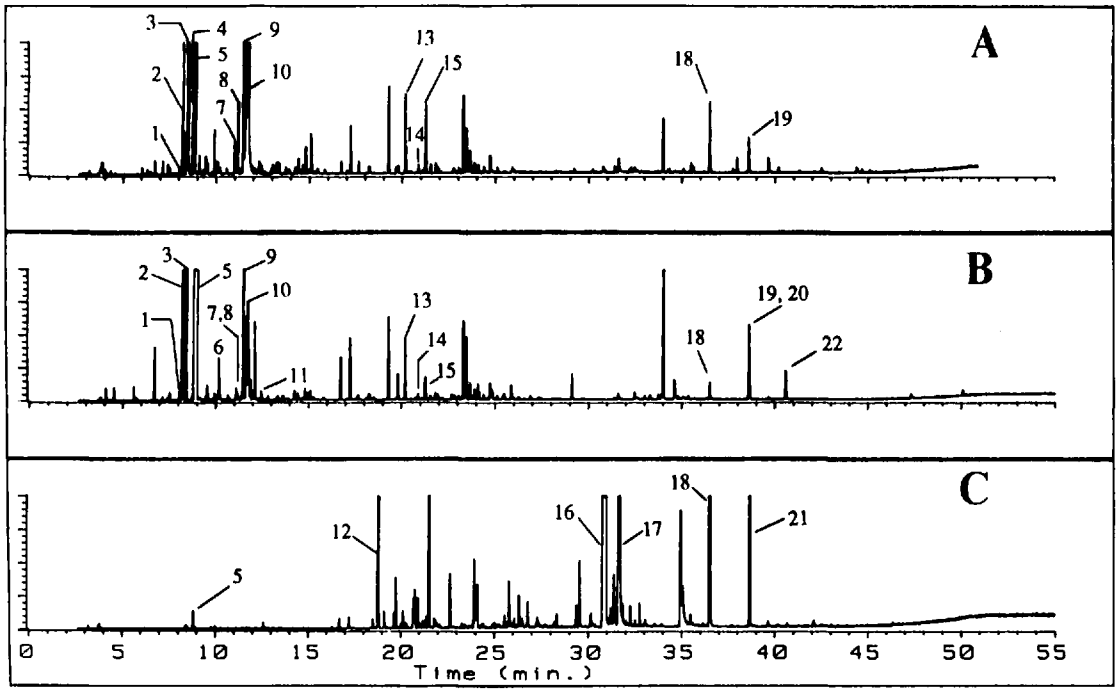

Fig. 2. Plots of gas chromatograms of the extracts of *Tagetes minuta*. (A) Floral extract. (B) Foliar extract. (C) Root extract. Numbered peaks are identified in Table 1.

min) than extracts for either aerial portion (Fig. 2A and B). Also, the floral extract (Fig. 2A) contained more α-terthienyl (peak 18) than the foliar extract (Fig. 2B), in addition to having a higher number of terpenoids with low molecular weights (peaks with retention times <15 min). Extract yields also varied for the three tissues: the flower yield was 0.68 ± 0.01%, the foliar yield was 0.28 ± 0.05%, and the root yield was 0.12 ± 0.02% (means ± SD for three replicates). A *T. minuta* plant harvested at full bloom has a mean biomass of 3.31 kg, of which 11% is flower,

Table 1. List of chemical components of floral, foliar, and root extracts of *T. minuta* tentatively identified by gas chromatography/mass spectroscopy

Peak no.	Compound name	% composition by area		
		Flower	Foliage	Root
1	Para-cymene	0.1	0.2	—
2	Limonene	3.8	9.6	—
3	Cis-ocimene	31.9	2.6	—
4	Trans-ocimene	1.3	—	—
5	Dihydrotagetone	13.5	47.5	0.2
6	Linalool	—	1.1	—
7	Alloocimene	0.8	—	—
7,8 (mixture)	Alloocimene, cis-epoxy-ocimene	—	0.3	—
8	Cis-epoxy-ocimene	0.4	—	—
9	Cis-tagetone	5.6	4.6	—
10	Trans-tagetone	19.1	6.0	—
11	Terpinen-4-ol	0.4	—	—
12	Alpha-gurjunene	—	—	7.0
13	Alpha-humulene	2.0	1.5	—
14	Germacrene-D	0.1	0.1	—
15	Germacrene-B	2.1	0.8	—
16	5-(But-3-ene-1-inyl)-2,2′-bithiophene	—	—	42.9
17	Palmitic acid	—	—	10.6
18	Alpha-terthienyl	2.1	0.4	9.9
19	5-Methyl-2,2′,2″-terthiophene	1.0	—	—
19,20 (mixture)	5-Methyl-2,2′,2″-terthiophene, 5-(4-Acetoxy-1-butenyl)-2,2′-bithiophene	—	2.3	—
21	5-(4-Acetoxy-1-butenyl)-2,2′-bithiophene	—	—	3.6
22	Unknown thiophene	—	0.7	—

Table 2. Probit regressions on pooled mortality and moribundity data from bioassays using three extracts from *T. minuta* against *Z. subfasciatus* at 24 and 48 h

Plant extract	Sex[a]	24 h			48 h		
		Slope ± SEM	LC_{50}[b]	95% CL	Slope ± SEM	LC_{50}[b]	95% CL
Flower	♂♂	3.68 ± 0.66	265.7	(175.9–343.1)	3.31 ± 0.54	245.4	(166.9–319.4)
	♀♀	4.27 ± 1.33	296.6	(108.4–395.9)	4.06 ± 1.03	267.3	(129.5–359.9)
Leaf	♂♂	6.18 ± 1.12	670.8	(575.4–762.0)	5.38 ± 1.08	646.1	(540.3–744.0)
	♀♀	5.66 ± 1.04	802.6	(707.0–916.7)	4.21 ± 1.01	787.2	(655.1–954.7)
Root	♂♂	3.94 ± 0.56	137.9	(107.5–165.3)	—	—	—
	♀♀	1.92 ± 0.70	252.0	(103.3–614.4)	—	—	—

[a]Ten replicates of five males and five females of *Z. subfasciatus* (0–1 d after adult emergence).
[b]Units are $\mu g/cm^2$ of Whatman no. 1 filter paper.

82% is foliage, and 7% is root, which would yield 2.38 g of flower extract, 7.56 g of foliar extract, and 0.31 g of root extract.

Preliminary investigation with the three extracts indicated that the floral and foliar extracts had similar activity and could be compared by using similar doses and count intervals, whereas the root extract required lower concentrations (was more toxic) but was slower acting. Therefore, bioassay designs encompassed this a priori knowledge of innate differences in the extracts.

The insecticidal activity of the extracts of *T. minuta* varied for each tissue. The root extract was most active at 24 h to both male and female *Z. subfasciatus*, followed by the flower extract and the foliar extract (Table 2). However, the range of LC_{50} values for the three extracts spanned only a 5-fold range for the males and approximately a 3-fold range for the females at 24 h (Table 2). The range of 95% CL is <10-fold for both sexes together across all three extracts (Table 2). Prolonged exposure to both the flower and leaf extracts for another 24 h reduced the LC_{50} value by 20–30 $\mu g/cm^2$ for both sexes, and in every trial the females were less susceptible than the males (Table 2).

The IT_{50} values for the flower and leaf extracts indicate that both can be fast-acting insecticides (Table 3). The floral extract has higher activity than the leaf extract at the lower concentration (Table 3). The data for the 1,000 $\mu g/cm^2$ concentration shows that both sexes are susceptible to this concentration (Table 3). Also, the males are more susceptible than the females in this bioassay (Table 3).

The temporal component of the bioassay indicated that the root extract, which was of greater innate toxicity at 24 h, acted more slowly. The data were influenced by a sharp increase in efficacy from 18 to 24 h, before which little toxicity was observed at any concentration. This resulted in higher IT_{50} values than were found for the extracts from the aerial portions of the plant (Table 4). Also, males were more susceptible to the root extract than the females (Table 4).

Discussion

All *T. minuta* tissues tested contain insecticidal components that may be useful for control of stored-product insects. Both the floral and foliar extracts contain compounds that are volatile with a rapid, possibly fumigative mode of action as indicated by the IT_{50} values. The LC_{50} of the leaf extract was higher than that for the floral extract and indicates that the total complex of volatile compounds extracted from this tissue is less toxic than those extracted from the floral

Table 3. Time-concentration-mortality regressions for insect incapacitation (knockdown data [24 h] + pooled mortality and moribundity data at 24 and 48 h) from bioassays using floral and foliar extracts from *T. minuta* against *Z. subfasciatus*

Extract	Sex[a]	β ± SEM[b]	IT_{50}[c] for 500 $\mu g/cm^2$	SIT_{50}[c] for 500 $\mu g/cm^2$	IT_{50}[c] for 1,000 $\mu g/cm^2$	SIT_{50}[c] for 1,000 $\mu g/cm^2$	Scaled deviance[d]	df
Flower	♂♂	3.95 ± 0.62	4.75	2.96	2.29	2.28		
	♀♀	3.60 ± 0.71	9.87	6.45	2.81	2.79		
							67.74	70
Leaf	♂♂	4.19 ± 0.63	33.2	2.95	2.61	2.49		
	♀♀	4.57 ± 1.12	—	—	10.24	2.79		

[a]Ten replicates of five males and five females of *Z. subfasciatus* (0–1 d after adult emergence).
[b]β is the concentration parameter for each sex and extract. None of the values was significantly different at $P = 0.05$, so a common β of 4.02 ± 0.36 was used to interpolate times to incapacitation.
[c]IT_{50} and SIT_{50} values are mathematically interpolated estimates of time (h) required to incapacitate 50% of the test insects and 50% of the susceptible test insects, respectively, by the end of the trial. If IT_{50} is much greater than SIT_{50}, then a percentage of insects did not succumb before 48 h. Neither estimate was determined if total incapacitation at the end of the experiment was <50%.
[d]The model used allowed for an extract*sex*time interaction with a common effect of concentration. Scaled deviance was not significant at $P = 0.05$.

Table 4. Time-concentration-mortality regressions for insect incapacitation (knockdown data [<24 h] + pooled mortality and moribundity data at 24 h) from bioassays using root extract from *T. minuta* against *Z. subfasciatus*

Sex[a]	$\beta \pm$ SEM[b]	IT_{50}[c] for 250 $\mu g/cm^2$	SIT_{50}[c] for 250 $\mu g/cm^2$	IT_{50}[c] for 400 $\mu g/cm^2$	SIT_{50}[c] for 400 $\mu g/cm^2$	IT_{50}[c] for 500 $\mu g/cm^2$	SIT_{50}[c] for 500 $\mu g/cm^2$	Scaled deviance[d]	df
♂♂	3.37 ± 0.47	20.4	19.0	17.3	15.6	11.9	11.3	30.00	31
♀♀	3.37 ± 0.66	—	—	22.4	18.6	20.7	18.0		

[a]Ten replicates of five males and five females of *Z. subfasciatus* (0–1 d after adult emergence).

[b]β is the concentration parameter for each sex. The values were not significantly different at $P = 0.05$, so a common β of 3.37 ± 0.38 was used to interpolate times to incapacitation.

[c]IT_{50} and SIT_{50} values are mathematically interpolated estimates of time (h) required to incapacitate 50% of the test insects and 50% of the susceptible test insects, respectively, by the end of the trial. If IT_{50} is much greater than SIT_{50}, then a percentage of insects did not succumb before 24 h. Neither estimate was determined if total incapacitation at the end of the experiment was <50%.

[d]The model used allowed for a sex*time interaction with a common effect of concentration. Scaled deviance was not significant at $P = 0.05$.

tissue. The root extract contains chemicals that are slower acting with a different mortality:time relationship than the aerial tissue extracts. All bioassays were commenced during the eighth hour of the photophase in our bioassay room. Thus the actual diel cycle was 4:12:8 (L:D:L). It is likely that the pronounced enhancement in efficacy between 18 and 24 h is the cumulative result of photoactivation of toxins from the root extract. Further, α-terthienyl is known to be photoactivated (Arnason et al. 1981).

Previous bioassays with herbivorous insects demonstrated either oral or topical activity for photoactivated compounds (for example, Champagne et al. 1986; Iyengar et al. 1987, 1990). The standard bioassay with mosquito larvae (Arnason et al. 1981) involves both ingestion and contact. Our bioassay did not involve direct topical application, but only initial contact through the tarsi or via spiracular uptake. Disorientation occurs with increasing duration of exposure to all extracts, which leads to greater topical exposure as insects fall and have difficulty righting themselves. This may explain why the cumulative effect of photoactivation occurs so late in the assay. With mosquito larvae, only 0.5 h of exposure to 15 W/m^2 of near-UV is required to photoactivate the material after an initial 0.5 h dark exposure (Arnason et al. 1981). The mortality delay in our bioassay may be a function of time required to accumulate a sufficient photoactivated dosage, instead of slower-acting toxicity in vivo.

This requirement for both prolonged exposure and light makes the root extract the least practical material we tested for insecticidal activity in stored products, despite the low LC_{50} value. Light is a limited resource in a closed structure containing bulk-stored products. Simple modifications could insolate the uppermost layer of stored foodstuffs, but the material beneath this uppermost layer would remain in continual darkness. Therefore, as was suggested by Arnason et al. (1981), it may be more useful to use the photoactivatable components (i.e., α-terthienyl) for mosquito control. The root extract, which was the most photo-potentiated of the three extracts we tested, could be used for this purpose rather than for control of storage pests.

Our data support an earlier report of greater susceptibility of male *Z. subfasciatus* to plant-derived compounds (Weaver et al. 1991). *Z. subfasciatus* is dimorphic and the susceptibility is likely a function of the smaller size of the males. Howe & Currie (1964) reported that the mean weight of males is 60% of that of the females across a variety of rearing conditions. There is no known morphological difference in potential absorptive surfaces such as antennae, nor are there noticeable differences in the behavioral activity of the sexes. In all cases the effect of concentration on sexes influenced the intercept portion of the statistical model, not the slope. This is consistent with a size effect rather than an innate difference in mode of susceptibility. The analysis of the vulnerability of each sex is important because the larger females are less susceptible, thus an analysis independent of sex will be biased by the response of the susceptible males, resulting in a lower estimation of effective dose. This species lays more than half of its eggs within 48 h of emergence from the host dried legume at high temperatures, and adequate control depends on the rapid incapacitation of the females.

We selected a bioassay to compare the insecticidal activity of these tissue extracts when applied to a surface, simulating protective treatment of stored foodstuffs. All tested extracts would be usable products from an "insecticidal crop" despite varying toxicities. This is important because approximately six to eight times more foodstuff can be equivalently protected by a single plant yield of floral or foliar extract than for the more potent root extract. Even the harvested floral extract, which is nearly three times more potent than the foliar extract, will only equivalently treat 85% of the foodstuff that the least efficacious foliar extract can. These yield considerations likely influence the selection of

screening criteria for crops grown for insecticidal purposes.

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