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Zingiberene and Resistance to Colorado Potato Beetle in *Lycopersicon hirsutum* f. *hirsutum*

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Zingiberene was detected in foliage extracts of *Lycopersicon hirsutum* f. *hirsutum*. 2-Tridecanone was not present in *L. hirsutum* f. *hirsutum* but did occur in *L. hirsutum* f. *glabratum*. F_2's of *L. hirsutum* f. *glabratum* × *L. hirsutum* f. *hirsutum* segregated for the presence of zingiberene and 2-tridecanone. The occurrence of zingiberene in *L. hirsutum* f. *hirsutum* coincided with the sporadic appearance of resistance to Colorado potato beetle (*Leptinotarsa decemlineata*) in this subspecies. Resistance to Colorado potato beetle in the F_2's was correlated with zingiberene content and with 2-tridecanone content. Extracts of *L. hirsutum* f. *hirsutum* foliage were toxic to Colorado potato beetle larvae at zingiberene contents estimated at 12-25 μg/larva. Zingiberene content of *L. hirsutum* f. *hirsutum* leaflets was estimated to be from 160 to 250 μg/2-cm^2 leaflet.

Resistance to Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say, has been identified in *Lycopersicon hirsutum* f. *glabratum* C. H. Mull (*gla*) (Schalk and Stoner, 1976) and attributed to the presence in glandular trichomes of certain toxic compounds (Kennedy and Sorenson, 1985), specifically, the methyl ketone 2-tridecanone (Kennedy et al., 1985). 2-Tridecanone also provides resistance in *gla* to the tobacco hornworm (*Manduca sexta* L.) (Fery and Kennedy, 1987; Williams et al., 1980) and contributes to resistance to the tomato fruitworm (*Heliothis zea* Boddie) (Dimock and Kennedy, 1983). *Gla* trichomes also contain 2-undecanone, which is toxic to *Pieris brassicae* L. (Lundgren et al., 1985).

Lycopersicon hirsutum f. *hirsutum* Humb. and Bonpl. (*hir*) is similar to *gla* in that the two subspecies share comparable densities of type IV and type VI trichomes (Snyder and Hyatt, 1984), the predominant types of glandular trichomes in *Lycopersicon* (Luckwill, 1943). The response of trichome density to day length is also similar in *gla* and *hir*. Type IV densities increase and type VI densities decrease under short days in both (Snyder and Hyatt, 1984). However, *hir* differs from *gla* in several respects. *Hir* has very little 2-tridecanone (Fery and Kennedy, 1987; Soost et al., 1968; Lin et al., 1987; Lundgren et al., 1985), has less 2-undecanone (Lin et al., 1987; Lundgren et al., 1985; Soost et al. 1968) and more 2-dodecanone (Soost et al., 1968) than *gla*, and contains predominantly sesquiterpenoids, including two C_{15} compounds that are absent in *gla* (Snyder et al., 1987; Snyder and Hyatt, 1984). Lin et al. (1987) discerned differences between *gla* and *hir* for three types of sesquiterpenes. Sesquiterpene A occurred in *gla* but not in *hir* and sesquiterpenes B and C in *hir* but not *gla*. Trichome extracts containing sesquiterpene B were toxic to larvae of *Keiferia lycopersicella* and *Spodoptera exigua* (Lin et al., 1987). Lundgren et al. (1985) reported that their accession of *gla* contained the sesquiterpene zingiberene, which was not detected in the *hir* accession they examined, but was the predominating component of the volatile fraction of another *hir* accession (Andersson et al., 1980). Sesquiterpenes have been implicated as insect antifeedants (Frazier, 1986; Mabry and Gill, 1979), but also as ovipositional stimulants (Juvik et al., 1988).

Hir, though resistant to a number of arthropod pests (Carter and Snyder, 1985; Juvik et al., 1982), is reportedly susceptible to CPB (Fery and Kennedy, 1987). However, an *hir* accession with which we have worked has exhibited sporadic resistance to CPB (Carter, 1987). Our objectives were to compare CPB resistance of this *hir* accession to that of *gla*, to analyze the inheritance of resistance in their segregating progeny, and to identify the factors conferring resistance to CPB in *hir*.

MATERIALS AND METHODS

Plant Material. Seeds of *L. hirsutum* f. *glabratum* C. H. Mull PI 134417 (*gla*) and *L. hirsutum* f. *hirsutum* Humb. and Bonpl. PI 126445 (*hir*) were obtained from the North Central Regional Plant Introduction Station at Ames, IA. *Gla* and *hir* were grown in the greenhouse at 16-30 °C and 14 h light/10 h dark. Crosses were made of *gla* as female parent and *hir* as the male. The *gla* × *hir* F_2 seeds were germinated in the greenhouse in spring 1986, and *gla* and *hir* were propagated by cuttings. The parent clones and F_2 seedlings were transplanted to the field in a completely randomized design in May 1986. The individual parent and F_2 plants were propagated by cuttings, grown in the greenhouse, and assayed again in May 1987.

Chemical Extraction and Identification. Foliage extracts were obtained in September 1986. Ten leaflets (ca. 2 cm^2 each) from upper leaves of each plant were extracted in 10 mL of distilled hexane for 2 h. Each sample

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was filtered through an 0.2-μm PTFE membrane filter (Millex-FGS, Millipore). Methyl ketones were detected by modification of the method of Nienhuis et al. (1985). For each sample, 0.2 mL of the hexane extract was added to 3 mL of 0.01% 2,4-dinitrophenylhydrazine (DNPH). This was vortexed 5 s and allowed to stand for 1 h, after which 0.3 mL of 5 N NaOH was added, and the solution vortexed again before the absorbance was read at 540 nm. The presence of 2-tridecanone was confirmed by analysis of the hexane extracts on a Varian Model 3700 gas chromatograph (GC) equipped with a flame ionization detector, using a 2 mm (i.d.) × 183 cm glass column packed with 2% OV-17 and programmed from 90 to 200 °C at 15 °C/min. Retention times and peak areas were determined on a Hewlett-Packard Model 3390A integrator. 2-Tridecanone was detected and quantified in the extracts by comparison to the peak retention time and peak area of a 2-tridecanone standard (Pfaltz and Bauer).

Zingiberene was identified in the hexane extracts of *hir* by capillary gas chromatography–mass spectrometry (GC–MS) using a 12 m × 0.2 mm column coated with HP-1 cross-linked methyl silicone gum. Mass spectra were obtained at an ionization potential of 70 eV, and the mass range of 40–250 amu was scanned repetitively at 2.04 scans/s. The presence of zingiberene in the F_2's was determined by GC retention time in comparison to the zingiberene peak in *hir* and the 2-tridecanone standard. The approximate quantity of zingiberene in *hir* extracts was estimated by comparison with the peak area of the 2-tridecanone standard. The amount of zingiberene in the F_2 extracts was only estimated relative to that in *hir*, i.e., by the F_2 zingiberene peak area as a percentage of the *hir* zingiberene peak area.

Ginger root extracts were made by grinding 50-g fresh weight of the roots of ginger (*Zingiber officinale* Roscoe) in 150 mL of distilled hexane, in a Virtis homogenizer. The homogenate was vacuum-filtered through Celite and then through an 0.2-μm PTFE membrane filter. The extract was analyzed by GC–MS, as described above.

Insect Assays. Resistance of intact *gla* and *hir* foliage to CPB was assayed three times: spring 1985, fall 1986, and spring 1987. The *gla* × *hir* F_2's were assayed in fall 1986 and in spring 1987. In each experiment, leaflets from the third to fifth node from the apex were placed on moist filter paper in Petri plates, three replicates per plant. Ten first-instar larvae (from eggs laid by adult CPB reared in the greenhouse on *Solanum tuberosum* cv. Katahdin) were placed on the foliage in each plate. Larval survival and the number of larvae feeding were determined each day for 5 days. The *L. esculentum* Mill. cv. Nova (*esc*) (Stokes Seeds) was used as the control in all CPB assays.

To assay the effects of *hir* and *gla* foliage extracts on CPB, hexane extracts collected in September 1987 were evaporated and redissolved in 100% ethanol at concentrations varying from 0 to 10 μg of 2-tridecanone/μL for the *gla* extracts and from 0 to 25 μg of zingiberene/μL for the *hir* extracts. The 2-tridecanone standard at 10 μg/μL and the ginger root extract at 15 μg of zingiberene/μL were also prepared for comparison. Ten first-instar CPB larvae were placed on Katahdin foliage in each of five petri plates per concentration. A 10-μL syringe was used to deliver a 1-μL drop of extract to each larva. Mortality was recorded after 24 h.

RESULTS AND DISCUSSION

2-Tridecanone occurred in foliage extracts of *gla* at about 300 μg/leaflet in fall 1986, at 160 μg/leaflet (0.05% of fresh weight) in spring 1987, and at 600 μg/leaflet (0.18% of fresh weight) in fall 1987. These values are comparable to the 180 μg of 2-tridecanone/*gla* leaflet found by Dimock and Kennedy (4), and to the 0.15–0.35% of *gla* foliage fresh weight detected by Fery and Kennedy (1987). 2-Tridecanone was not detected in *hir* extracts at any time.

Table I. Mass Spectra of the Major Peak in *L. hirsutum* f. *hirsutum* (*hir*) Extracts and the Corresponding Peak in Ginger Root and of Zingiberene

compound	*m/z* (% relative intensity)[a]				
hir major peak	204 (6)	119 (94)	93 (100)	91 (53)	77 (48)
	69 (46)	55 (23)	56 (21)	41 (87)	
ginger root	204 (7)	119 (94)	93 (100)	91 (51)	77 (44)
	69 (44)	56 (20)	55 (21)	41 (80)	
L-zingiberene[b]	204 (8)	119 (79)	93 (100)	91 (26)	77 (26)
	69 (40)	56 (18)	55 (17)	41 (44)	

[a] The *m/z* values are arranged in order of descending fragment size. [b] Mass Spectrometry Data Center, 1983.

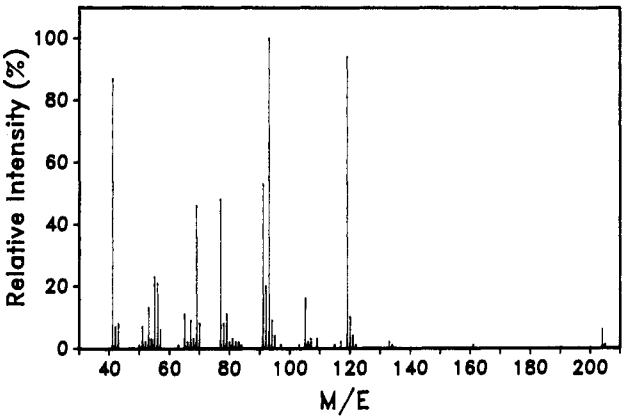

Figure 1. Mass spectrum of the major peak in *hir*. Height of the vertical lines is proportional to the relative intensity of the major fragment peaks compared to the base peak at 100. Ionizing potential of 70 eV, 2.04 scans/s.

The major peak in the gas chromatogram of the *hir* extract had a slightly shorter retention time than 2-tridecanone and was confirmed as a separate peak by augmentation with the 2-tridecanone standard. GC–MS analysis of the *hir* peak indicatd a molecular ion, 204 (6), identical with that of zingiberene, and close similarities to the major ion fragments and relative intensities of other published spectra of L-zingiberene (1,3-cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-[*S*(*R**,*S**)] (Hirose, 1965; Moshonas and Lund, 1970; Mass Spectrometry Data Center, 1983) (Table I). These major ion fragments and relative intensities also appear to correspond to those of the compound designated as sesquiterpene B by Lin et al. (1987). The mass spectrum of the *hir* major peak corresponding to zingiberene is shown in Figure 1. The *hir* extract also contained smaller amounts of several other probable sesquiterpenes. The largest of these occurred at 15% of the zingiberene concentration and displayed a mass spectrum similar to that of curcumene (Mass Spectrometry Data Center, 1983).

In the ginger root extract, the peak corresponding to the major peak in the *hir* extract was identified by GC retention time and by augmentation with the *hir* extract. GC–MS analysis of this peak indicated correspondence to the putative zingiberene peak in *hir* (Table I). Zingiberene was reported as the major peak in the essential oil of ginger root by Nigam et al. (1964). The ginger root extract also contained a sesquiterpene, possibly curcumene, at 10% of the zingiberene peak area, and three other sesquiterpenes not detected in *hir*.

Zingiberene occurred in foliage extracts of *hir* at about 160 μg/leaflet in fall 1986 and at 250 μg/leaflet (270 μg/g fresh weight) in fall 1987. Zingiberene was not detected

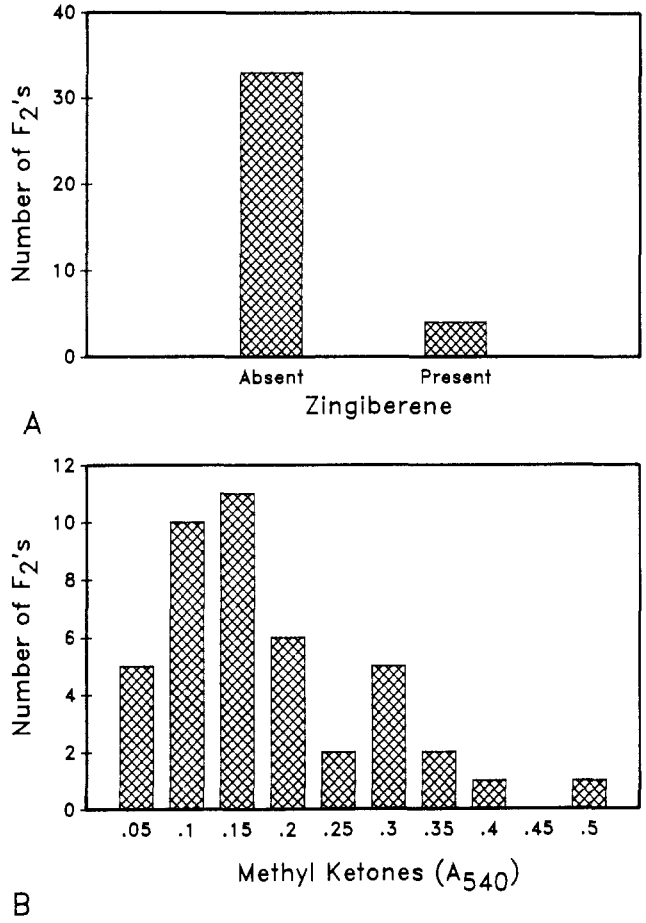

Figure 2. *Gla* × *hir* F_2 segregation for zingiberene and methyl ketones: (A) zingiberene content, $X^2(3:1) = 3.57$ $p > 0.10$, $X^2(15:1) = 0.81$, $p > 0.50$; (B) methyl ketone content, as absorbance at 540 nm (A_{540}), $X^2(63:1) = 0.444$, $p > 0.70$.

in *hir* extracts collected in spring 1987 nor in extracts of *gla* foliage at any time. Lundgren et al. (1985) found 1.3 μg of zingiberene/mg of foliage fresh weight of a *gla* accession LA 1223. Lin et al. (1987) found 2–4 ng per type VI trichome gland and 2–3 glands/mm² in *L hirsutum* accession LA 361. At 3 type VI trichomes/mm², this would amount to about 8 ng/mm² of leaflet surface or, for both surfaces of a 2-cm² leaflet, to 3 μg/leaflet, considerably less than in our material. However, Snyder et al. (1987) found an average of 1.75 nmol of sesquiterpenes/mm² in four accessions of *hir*. For both surfaces of leaflets of 2 cm², the approximate area of the leaflets in our foliar extracts, this would amount to 143 μg/leaflet, which is close to our estimates of zingiberene content.

The peak corresponding to zingiberene was detected in 4 of the 37 F_2's examined and occurred at 80% to over 300% of the amount in the *hir* parent, based on relative peak areas. F_2 segregation for presence or absence of zingiberene did not differ significantly from either a one- or two-gene model ($X^2 = 3.6$, $p > 0.10$ and $X^2 = 0.81$, $p > 0.50$, respectively) (Figure 2A). Segregation for the presence of methyl ketones (Figure 2B) suggested a 63:1 ratio ($X^2 = 0.044$, $p > 0.70$), which fits a three-gene model where methyl ketone content comparable to *gla* was conditioned by homozygous recessive alleles at three loci, as described by Fery and Kennedy (1987).

Percent survival of CPB larvae was compared among *gla*, *hir*, and *esc* on several occasions (Table II). CPB larval survival assayed on *hir* in spring 1985 and 1987 was comparable to that on the susceptible *esc* cultivar. However, in fall 1986, the performance of *hir*, which was usually

Table II. Percent CPB Survival after 3 Days on *L. hirsutum* f. *glabratum* (*gla*), *L. hirsutum* f. *hirsutum* (*hir*), and *L. esculentum* (*esc*) in Spring 1985, Fall 1986, and Spring 1987

species	% CPB survival[a] spring 1985	fall 1986	spring 1987
gla	27 a	27 a	17 a
hir	81 b	5 a	88 b
esc	97 b	85 b	87 b

[a] Within each column, means followed by the same letter do not differ at $p < 0.05$, by the Student's *t*-test.

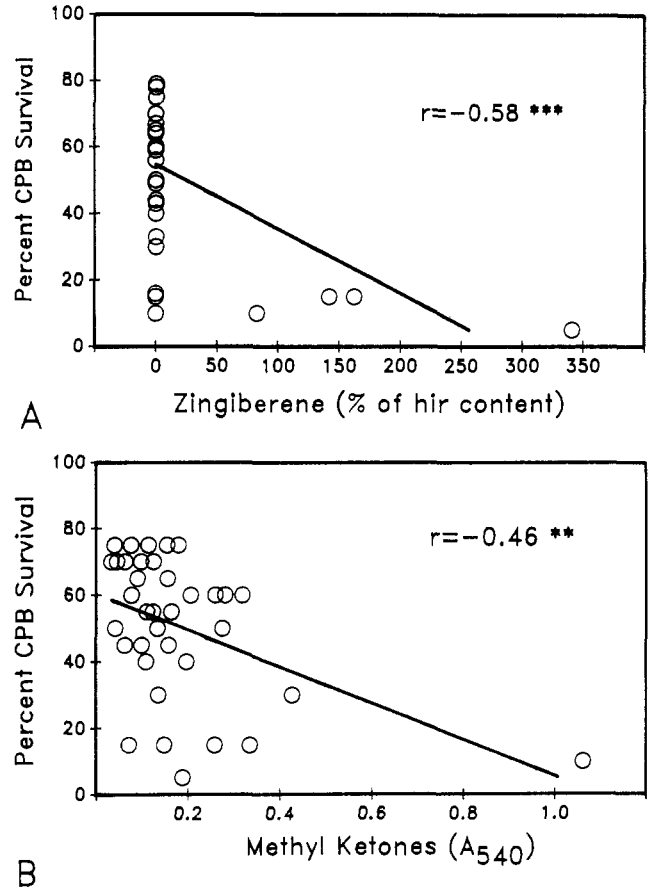

Figure 3. Percent CPB survival in relation to zingiberene or methyl ketone content of *gla* × *hir* F_2's: (A) percent CPB survival (% S) in relation to F_2 zingiberene content (Z), as a percentage of the zingiberene peak area of *hir*, % S = 55 − 0.18(Z), $R^2 = 0.33$, $p < 0.001$; (B) percent CPB survival (% S) in relation to F_2 methyl ketone content (MK), as absorbance at 540 nm (A_{540}), % S = 60 − 55(MK), $R^2 = 0.20$, $p < 0.01$.

susceptible to CPB, was atypical. CPB survival on *hir* was significantly less than on *esc* and comparable to that on *gla* in this assay. Kennedy et al. (1981) found that trichome densities, 2-tridecanone content, and tobacco hornworm mortality of *gla* were lower under short days than under long days. Similar effects might lead to reduced CPB resistance on *gla* under the short days of fall. However, this does explain the significantly greater mortality on *hir* compared to *esc* in the fall assay.

CPB survival on the F_2's assayed in fall 1986 ranged from 10% to 80% and declined in relation to zingiberene content (as a percent of zingiberene peak area of *hir*) (Figure 3A) ($R^2 = 0.33$, $p < 0.001$). CPB survival also declined as methyl ketones increased (Figure 3B), although the coefficient of determination for this relationship was somewhat lower ($R^2 = 0.20$, $p < 0.001$). When CPB survival was regressed against both relative zingiberene content and methyl ketone content, these two variables ac-

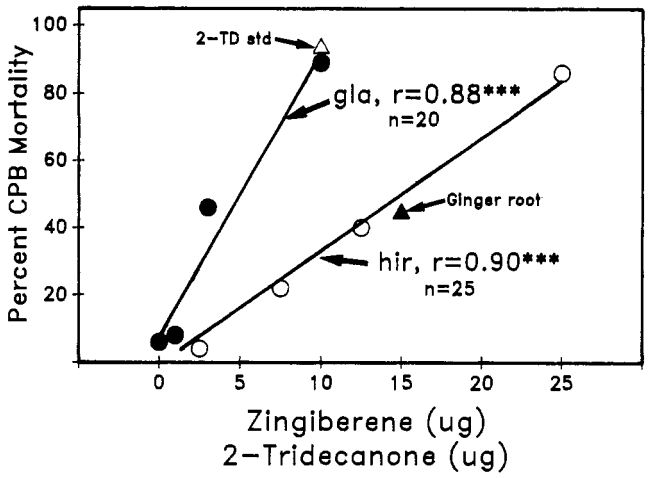

Figure 4. Percent CPB mortality (% M) in relation to zingiberene content in *hir* extracts or to 2-tridecanone content in *gla* extracts; and % M at 10 $\mu g/\mu L$ of the 2-tridecanone standard (2-TD std) or at 15 $\mu g/\mu L$ zingiberene in ginger root extracts. Each point represents the mean of five observations. *Hir* extracts: % M = 0 + 3.4(zingiberene) (μL), $R^2 = 0.79$, $p < 0.001$. *Gla* extracts: % M = 8 + 8.6(2-tridecanone) (μL), $R^2 = 0.78$, $p < 0.001$.

counted for 53% of the variation in CPB survival (R^2 = 0.53 , $p < 0.001$).

In spring 1987, when the CPB assay of *gla* × *hir* F_2's was repeated, CPB survival ranged from 60% to 100%. None of the F_2's displayed CPB resistance comparable to that of *gla* or to that which occurred on *hir* and some of these same F_2's in the previous fall assay. Zingiberene and methyl ketone contents were not determined in spring 1987. However, as zingiberene was not detected in *hir* at this time, it is probable that it was not present in the F_2's as well.

Crude hexane extracts of both *gla* and *hir* were toxic to CPB larvae (Figure 4). CPB mortality increased in relation to tridecanone concentration in the *gla* extracts and in relation to zingiberene content in the *hir* extracts. Although other compounds were also present in the extracts, zingiberene accounted for over 40% of the cumulative areas of the peaks detected at the highest concentration of the *hir* extract, and its peak area was from 3 to 100 times greater than that of any other peak. The ginger root extract, which contained about 15 $\mu g/\mu L$ zingiberene, was also as toxic to the larvae as the *hir* extracts with roughly equivalent amounts of zingiberene (Figure 3).

CONCLUSIONS

The concurrent appearance of zingiberene and CPB resistance in *hir* and the association of CPB resistance with zingiberene in the F_2's strongly suggest an association between zingiberene and CPB mortality. *Hir* extracts containing predominantly zingiberene were toxic to CPB larvae at concentrations of approximately 15–25 μg/larva. In our *hir* accession, the concentration of zingiberene in young leaflets, approximately 2 cm^2, was estimated at 160–250 μg/leaflet. If zingiberene is present mostly in the glandular trichome exudates, as suggested by Lin et al. (1987), then on our leaflets the concentration of zingiberene could be 0.4–0.6 $\mu g/mm^2$ per leaflet surface. If a first-instar larva is exposed to, e.g., 20 mm^2 of one leaflet surface (ca. one-tenth of one surface of a leaflet), then that larva might encounter from 8 to 12 μg of zingiberene, close to the toxic levels of zingiberene in the *hir* extract. However, the leaflets directly assayed for CPB survival were larger than those from which extracts were obtained and may be expected to have lower trichome densities (Snyder and Carter, 1984). Consequently, the amount of zingiberene per unit of surface area on these leaflets may have been lower than these estimates. We are in the process of purifying zingiberene from the roots of ginger (*Z. officinale*) for further assays of its potential toxicity to CPB and to improve the accuracy of zingiberene quantification in *hir*.

The presence of zingiberene in *hir* was not constant. Trichome densities and 2-tridecanone content are known to fluctuate with environmental factors such as day length and/or temperature (Kennedy et al., 1981; Snyder and Hyatt, 1984), which may also affect zingiberene content. If zingiberene does contribute to CPB resistance, then a short-day requirement, e.g, for its expression may be a problem for practical use in the field. Inheritance of zingiberene may also be more complex in the genotypic background of *L. esculentum*. Further explorations of environmental and genetic effects on zingiberene synthesis are required for evaluation of the potential of zingiberene for breeding for CPB resistance in tomato.

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Registry No. Zingiberene, 495-60-3; 2-tridecanone, 593-08-8.

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Distribution and Elimination of [^{14}C]Malathion in the Rat

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The distribution pattern of malathion after intravenous administration of [^{14}C]malathion to rats was studied by whole-body autoradiography. Highest levels of radioactivity were detected in the liver and the kidney, which reached peak values 1–3 min following administration. The amount of radioactivity decreased rapidly, and after 24 h, only low levels in the liver, the kidney, the intestines, and the Harderian gland were detected.

The organophosphorus compound malathion, *O,O*-dimethyl *S*-(1,2-dicarbethoxyethyl) phosphorodithioate, has, compared to other organophosphates, a low mammalian toxicity and is widely used as a broad-spectrum contact insecticide and acaricide, as well as an ectoparasitic agent on both animals and human beings. Some investigations on the pharmacokinetic properties of malathion are published (Bourke et al., 1968; Gupta and Paul, 1976; Muan et al., 1985), but little has been done to clarify the distribution pattern of the compound. In the present work, the distribution in rats after intravenous administration of malathion has been studied by whole-body autoradiography and liquid scintillation counting.

MATERIALS AND METHODS

Test Materials. [^{14}C]Malathion, with specific activity 112 μCi/mg, was obtained from Amersham International plc (Buckinghamshire, England). It was prepared by the condensation of diethyl [2,3-$^{14}C_2$]maleate with *O,O*-dimethyl dithiophosphoric acid, and the radiochemical purity was 98%, as determined by thin-layer chromatography on silica gel in the manufacturer's laboratory. A 250-μCi portion of [^{14}C]malathion was dissolved in 1 mL of 96% ethanol and a salt buffer to 5 mL, to a final concentration of 50 μCi/mL.

Test Animals and Administration. Eight male Wistar rats (Møllegaard, Denmark) weighing 170–220 g were used. The animals were kept in cages, three in each, and maintained in 12 h light and 12 h darkness at 20 °C and 55% relative humidity. They were fed ad libitum with a commercial pelleted diet containing 24% proteins, 2% fat, and minerals and vitamins in adequate amounts (Møllesentralen I/S, P. Larsen & Co., Oslo, Norway) and had free access to water during the experiment.

The rats were given 200 μL/100 g of body weight (bw) of the solution of [^{14}C]malathion in the tail vein, corresponding to 10 μCi/100 g bw or 0.09 mg of malathion/100 g bw.

Whole-Body Autoradiography. One rat was sacrificed with diethyl ether euthanasia at 1, 3, and 10 min and 1, 2, 6, 12, and 24 h after administration. The animals were mounted in a 1% (w/v) aqueous gel of carboxymethylcellulose and frozen in a bath of *n*-hexane cooled with solid carbon dioxide to about −75 °C.

Sagittal sections (30 μm) through the whole frozen animal containing representative samples of all tissues were taken at different levels of the body. Three successive sections at each level were collected on tape (No. 821, 3 M Co., St. Paul, MN) at −20 °C in a PMV cryomicrotome (PMV 450 MP, Stockholm, Sweden). The sections were

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