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Sesquiterpenes in Glandular Trichomes of a Wild Tomato Species and Toxicity to the Colorado Potato Beetle

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Zingiberene, a sesquiterpene associated with resistance to the Colorado potato beetle (*Leptinotarsa decemlineata* Say) in *Lycopersicon hirsutum* f. *hirsutum* Humb. and Bonpl., occurred in the glandular tips of the type VI trichomes of this tomato species and was not present in the type IV or other trichomes or in the leaf matrix. Thus, transfer of the capacity to accumulate zingiberene into the cultivated tomato (which already has type VIs but lacks type IVs) will not be complicated by a requirement for multiple genes conferring high type IV densities. An alternative source of sesquiterpenes, the essential oil from roots of ginger (*Zingiber officinale* Roscoe), provided an extract containing predominantly zingiberene and smaller amounts of two sesquiterpenes tentatively identified as curcumene and bisabolene, in quantities sufficient for bioassays. This extract was toxic to CPB larvae at an LD_{50} at 7 μg of sesquiterpene/larva, a level provided by type VI trichomes occupying only 10–20 mm^2 of leaflet surface.

Lycopersicon hirsutum f. *hirsutum* Humb. and Bonpl. (*hir*) is resistant to a number of arthropod pests (Carter and Snyder, 1985; Juvik et al., 1982; Rick, 1982) but is reportedly susceptible to Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Fery and Kennedy, 1987). However, an *hir* accession with which we have worked exhibits sporadic resistance to CPB. Our *hir* does not contain the methyl ketone 2-tridecanone, which confers resistance to CPB and other insects in the related subspecies *L. hirsutum* f. *glabratum* C.H. Mull (*gla*) (Kennedy and Sorenson, 1985), but we have identified the sesquiterpene zingiberene in foliage extracts of *hir* by GC–MS (Carter et al., 1989). Zingiberene is occasionally found at high levels in *hir* leaves (Carter et al., 1989; Snyder et al., 1987) and appears to confer CPB resistance to *hir* and to *gla* × *hir* progeny segregating for zingiberene and tridecanone contents (Carter et al., 1989). Zingiberene content is variable in *hir* (Carter et al., 1989; Snyder and Hyatt, 1984b). However, zingiberene and related sesquiterpenes occur as major components of ginger root essential oil (Chen and Ho, 1988; Nigam et al., 1984), which would be a convenient source of sesquiterpenes for bioassay on CPB.

The location of terpenes implicated in insect resistance has often been ascribed to glandular trichomes located on the leaf surfaces of many plants (Duffey, 1986; Levin, 1973; Loomis and Croteau, 1980). Several types of trichomes are present on leaves of *Lycopersicon*, and species differ with respect to the types and densities of trichomes. For example, the type VI trichome, which has a four-celled glandular tip, is present at comparable densities on leaves of *hir*, *gla*, and the cultivated tomato, *Lycopersicon esculentum* (Mill.) (*esc*); but the type IV trichome, having a single glandular tip cell, is present on leaves of *hir* and *gla* but not on *esc* (Luckwill, 1943; Snyder and Carter, 1984a). Unidentified sesquiterpenes were found in the type VI trichomes of *L. hirsutum* (Lin et al., 1987), but at much lower concentrations per leaf than we have found (Carter et al., 1989). Snyder and Hyatt (1984) assigned zingiberene to type VI trichomes but did not distinguish between type VI and other trichomes in the collection of their extracts. Knowledge of the specific trichome in which zingiberene resides is important in transferring high zingiberene contents to the cultivated tomato, because if zingiberene occurs in the type VI trichome, rather than or in addition to the ubiquitous type VI, the transfer of zingiberene from *hir* to *esc* would be complicated by the necessity to transfer multiple genes conferring high type IV densities (Carter and Snyder, 1986). Thus, it is important to determine the specific trichome(s) in which zingiberene accumulates.

Our objectives were to determine whether zingiberene occurs specifically in *hir* trichomes and, if so, in which class of trichomes, and to assay the effect of zingiberene and

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Table I. Mass Spectra and Retention Indices (I) of the Three Sesquiterpene Peaks (Peak B at 66%, Peak C at 33%, Peak A at 1%) in the Ginger Root Extract and Corresponding Spectra of L-Zingiberene, Bisabolene, and α-Curcumene

compound	I[a]	m/z (% relative intensity)[b]					
peak B (66%)	1454	204 (7)	119 (94)	93 (100)	91 (51)	77 (44)	
		69 (44)	56 (20)	55 (21)	41 (80)		
L-zingiberene[c]		204 (8)	119 (79)	93 (100)	91 (26)	77 (26)	
		69 (40)	56 (18)	55 (17)	41 (44)		
peak C (33%)	1461	204 (9)	161 (21)	109 (14)	94 (7)	93 (47)	92 (26)
		91 (41)	79 (19)	77 (34)	69 (95)	55 (29)	41 (100)
bisabolene[c]		204 (32)	109 (24)	94 (28)	93 (64)		
		79 (28)	69 (86)	55 (28)	41 (100)		
β_2-bisabolene[d]		204 (21)	93 (56)	69 (76)	41 (100)		
peak A (1%)	1447	202 (19)	145 (19)	132 (66)	131 (26)	120 (32)	119 (100)
		105 (86)	91 (70)	79 (34)	77 (37)	55 (42)	41 (94)
α-curcumene[c]		202 (27)	145 (24)	132 (73)	131 (23)	120 (25)	119 (100)
		41 (36)					

[a] Kovats, 1958. [b] The m/z values are arranged in order of descending fragment size. [c] Mass Spectrometry Data Center, 1983. [d] Hirose, 1965.

other sesquiterpenes from ginger root on CPB.

MATERIALS AND METHODS

L. hirsutum f. *hirsutum* PI 126445 (obtained originally from the North Central Regional Plant Introduction Station, Ames, IA) was propagated by sib crosses and by cuttings and grown in a shaded greenhouse under natural light of approximately 14-h light/10-h dark and temperatures of 24–38 °C. For analysis of trichome contents, leaflets from the third to fifth nodes from the apex of a clone selected for high sesquiterpene content were weighed and the petioles placed in tap water. The glandular tips of the type VI trichomes were removed from both surfaces of individual leaflets with a 10-μL syringe needle repeatedly dipped in hexane, while the leaflet surface was observed under a dissecting microscope. All other trichomes were left undisturbed. After all type VI tips were removed, the leaflet area was determined with a Li-Cor LI-3000 portable leaf area meter, and the leaflet was placed in 10 mL of hexane overnight. Following extraction of the type VI trichome tips and the leaflet surface, leaflets were ground in hexane and extracts of the interior matrix filtered through an 0.2-μm nylon membrane.

The hexane extracts of type VI tips and hexane extracts of the leaflet surface and interior were evaporated to dryness under nitrogen and redissolved in 50 μL of hexane. Sesquiterpenes were identified by gas chromatography–mass spectroscopy (GC–MS), as previously described (Carter et al., 1989), 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, the mass range of 40–250 amu was scanned repetitively at 2.04 scans/s, and Kovats retention indices (Kovats, 1958) were calculated in comparison to a series of C_{12}–C_{18} n-alkane standards (Alltech).

Sesquiterpene contents of the extracts were quantified by comparison to the peak area of a farnesol (Sigma) standard, determined by GC on a Varian Model 3700 GC equipped with a flame ionization detector, using a 2 mm (i.d.) × 183 cm glass column packed with 2% OV-1m and programmed from 90 to 200 °C at 15 °C/min, determining retention time and peak areas with a Hewlett-Packard Model 3390A integrator.

Extracts of ginger root (*Zingiber officinale* Roscoe) were obtained by grinding fresh roots in distilled hexane (3 mL of hexane/g fresh weight of root tissue) on a Virtis homogenizer. The homogenate was filtered through Celite and then through an 0.2-μm PTFE membrane filter. The filtered extract was evaporated to dryness under nitrogen and redissolved in a convenient volume of acetonitrile. Partial purification was conducted by high-pressure liquid chromatography (HPLC), using two 57 cm × 6.5 mm preparative columns in series, both packed with C_{18} Porasil. With a flow rate of 5 mL/min, a 25-min gradient was established starting with an aqueous solution of 40% acetonitrile and ending with 100% acetonitrile. The peak containing zingiberene and closely related sesquiterpenes, as confirmed by GC, was fractionally collected. Sesquiterpenes were identified by GC–MS as described above.

The partially purified ginger root extract was concentrated under nitrogen and redissolved in ethanol for bioassay. Extracts were serially diluted in three replicates of concentrations ranging from 0 to 15 μg of sesquiterpenes/μL and topically applied to first-instar CPB larvae at 1 μL of solution/larva. There were 10 CPB larvae/replicate and 3 replicates/concentration. Mortality was determined after 24 h at 20 °C, and data were subjected to Probit analysis using Statistical Analysis Systems programs.

RESULTS AND DISCUSSION

Zingiberene occurred almost exclusively in the glandular tips of *hir* type VI trichomes (Figure 1A) and was undetectable in the remainder of the leaflet surface (Figure 1B), or interior matrix, at the limit of detectability by our methods, i.e., at <0.1 μg/cm^2 of leaflet surface. Thus, zingiberene apparently does not occur at substantial levels in any other trichome or in the leaflet matrix. The concentration of zingiberene for the combined adaxial and abaxial surface area of the representative leaflet of Figure 1 was 92 μg/cm^2. Zingiberene concentrations of other *hir* clones ranged from 10 to 107 μg/cm^2, and type VI densities averaged 8/mm^2 of single surface. At 50 μg of zingiberene/cm^2 of a single surface, each type VI tip would contain about 0.06 μg of zingiberene.

The accumulation of zingiberene in the type VI trichome and not in other trichomes is significant for the eventual transfer of zingiberene-based resistance to the cultivated tomato. The type VI trichome, in contrast to type IVs and some other trichomes, is present at comparable densities on leaves of both *hir* and the cultivated tomato, obviating the necessity to introduce genes for a new morphological structure or to select for multiple genes conferring high trichome densities. Indeed, inheritance of zingiberene accumulation in progeny of *gla* × *hir* was relatively simple (Carter et al., 1989), though analysis of the number of genes required in the interspecific cross to *esc* is clearly required before embarking upon transfer of zingiberene-based resistance to the cultivated tomato.

The partially purified ginger root extract was comprised overwhelmingly of three sesquiterpenes accounting for 66%, 33%, and 1% of the sesquiterpene peak area (Figure 2). The largest component was confirmed as zingiberene, and the other two peaks were tentatively identified as bisabolene and curcumene, by GC–MS analysis (Figure 3) and comparison to published spectra (Table I). Curcumene also occurs in *hir*, though at lower concentrations than zingiberene (Carter et al., 1989), and may result from decomposition of zingiberene in extracts exposed to light and air (Smith and Robinson, 1981). Bisabolene has not been detected in *hir* (Good and Snyder, 1988; Carter et al., 1989).

CPB mortality increased in relation to sesquiterpene concentration of the partially purified ginger root extract (Figure 4), giving an LD_{50} of 7.2 μg of sesquiterpene/CPB,

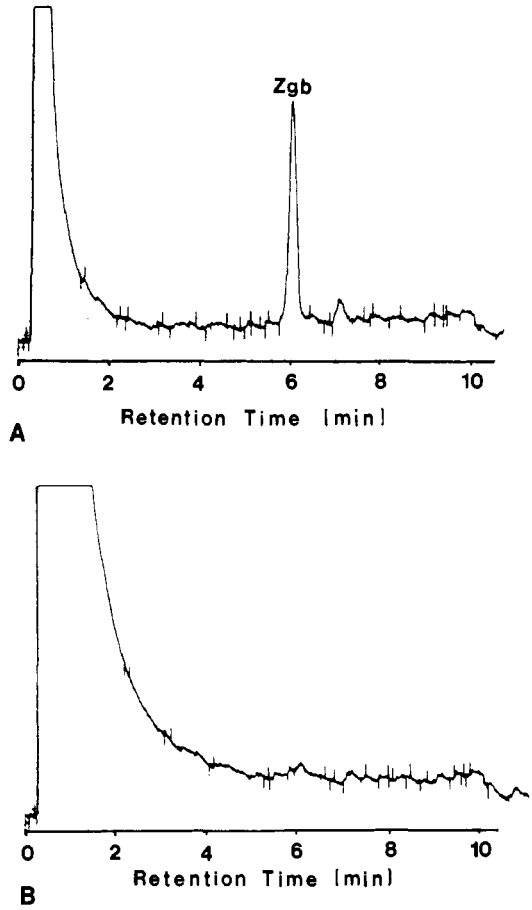

Figure 1. Gas chromatograms of *hir* extracts. Samples were chromatographed on a 2 mm (i.d.) × 183 cm glass column packed with 2% OV-17 using a program initiated at 90 °C for 2 min following injection and increasing by a gradient of 15 °C/min to 200 °C with a 1-min hold at the final temperature. Detection was by flame ionization. Key: (A) hexane extract (1 μL) of the glandular tips of the type VI trichomes of a *hir* leaflet, showing a zingiberene peak (Zgb) at 5.96 min; (B) hexane extract (3 μL) of the same leaflet following removal of all type VI trichome tips.

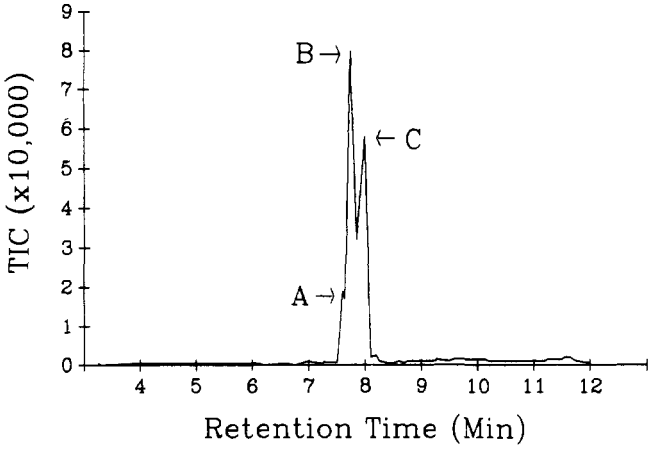

Figure 2. Total ion current (TIC) mass chromatogram of the partially purified ginger root extract. The largest peak (B) and two other major peaks (C and A) are 66%, 33%, and 1%, respectively, of the total sesquiterpene peak area.

with 95% fiducial limits of 6.5–8.1 μg. CPB mortality of the ethanol solvent controls averaged 3%. Although the assay did not distinguish among the three sesquiterpene compounds present, only zingiberene and curcumene are present in both ginger root and *hir*, and curcumene accounted for only 1% of the total sesquiterpene content of

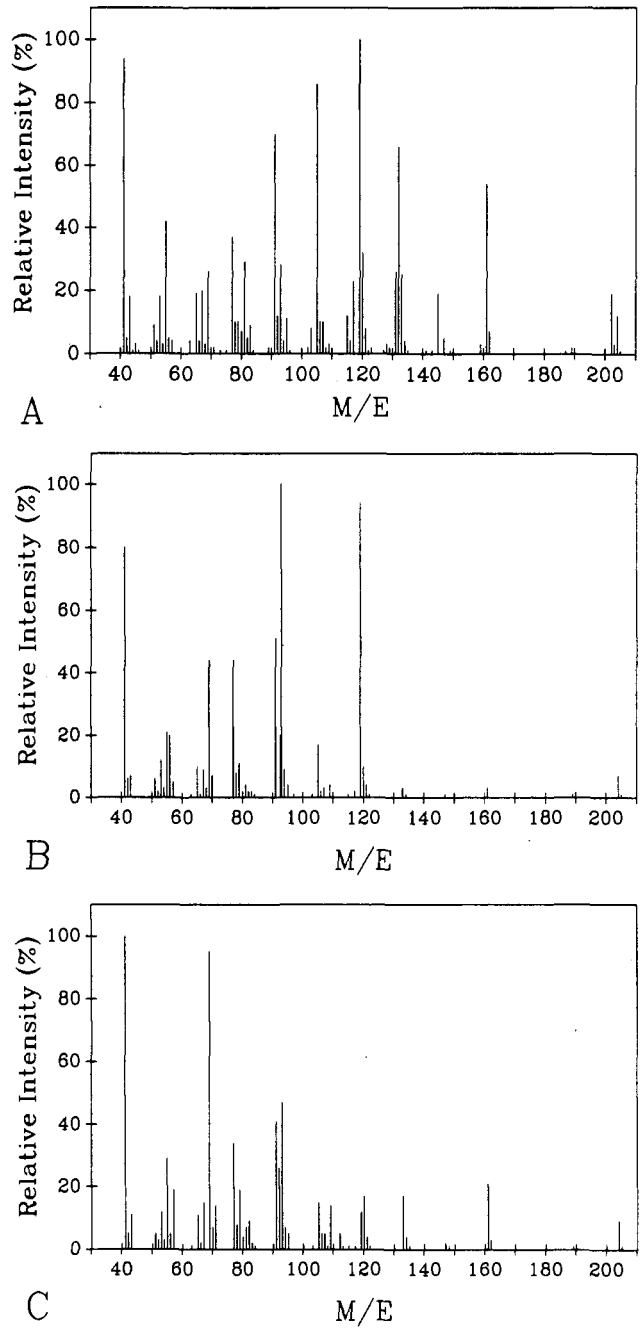

Figure 3. Mass spectra of the three sesquiterpene peaks of ginger extract. Height of the lines is proportional to the relative intensity of the major ion fragment peaks compared to the base peak at 100. Ionizing potential of 70 eV, 2.04 scans/s. Key: (A) smallest peak, tentatively identified as α-curcumene; (B) largest peak, identified as L-zingiberene; (C) second largest peak, tentatively identified as bisabolene.

the ginger root extract. If zingiberene alone were responsible for the toxicity of the extract, the LD_{50} would be approximately 5 μg of zingiberene/larva. If each *hir* type VI trichome tip contains 0.06 μg of zingiberene and there are 8 type VI tips/mm², a 10-mm² area of a single *hir* leaflet surface would be sufficient to provide 5 μg of zingiberene.

CONCLUSIONS

Zingiberene occurred exclusively in the type VI trichome tips of *hir*, at an average concentration of 60 ng/VI tip, or about 50 μg/cm² of a single surface at type VI densities of 8/mm². Thus, introduction of the ability to synthesize zingiberene into germplasm of the cultivated tomato will not be complicated by the necessity to select for high

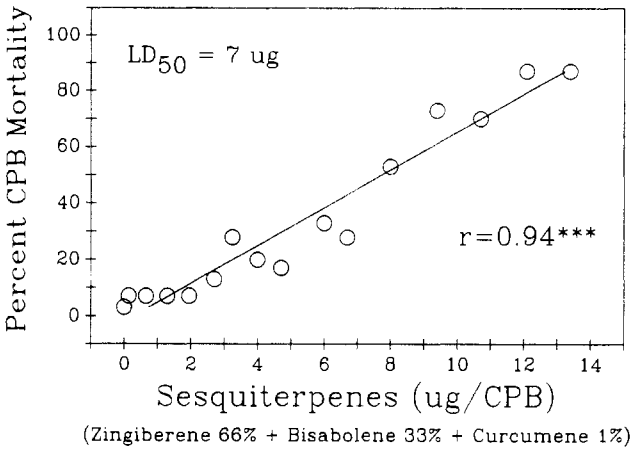

Figure 4. Percent CPB mortality (% M) in relation to sesquiterpene content (S) in ginger root extracts. % M = −3.7 + 6.7S (μg), $R^2 = 0.88$, $p < 0.001$.

densities of other trichomes.

Partially purified ginger root extracts contained predominantly zingiberene and smaller amounts of two other sesquiterpenes tentatively identified as curcumene and bisabolene. As purified, this mixture was toxic to CPB at an LD_{50} of 5 μg of zingiberene/larva, a level of zingiberene that can be supplied by type VI trichomes occupying 10 mm^2 of a single *hir* leaflet surface. Ginger root also provides an alternative source for the purification and bioassay of sesquiterpenes implicated as resistance factors in *hir*.

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Registry No. Zingiberene, 495-60-3; curcumene, 644-30-4; bisabolene, 495-62-5.

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