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Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plants

(beet armyworm/plant–insect interaction)

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ABSTRACT **Cotton plants attacked by herbivorous insect pests emit relatively large amounts of characteristic volatile terpenoids that have been implicated in the attraction of natural enemies of the herbivores. However, the composition of the blend of volatile terpenes released by the plants varies remarkably throughout the photoperiod. Some components are emitted in at least 10-fold greater quantities during the photophase than during the scotophase, whereas others are released continuously, without conforming to a pattern, during the entire time that the plants are under herbivore attack. The diurnal pattern of emission of volatile terpenoids was determined by collecting and analyzing the volatile compounds emitted by cotton plants subjected to feeding damage by beet armyworm larvae *in situ*. The damage was allowed to proceed for 3 days, and volatile emission was monitored continuously. During early stages of damage high levels of lipoxygenase-derived volatile compounds [e.g., (*Z*)-3-hexenal, (*Z*)-3-hexenyl acetate] and several terpene hydrocarbons [e.g., α-pinene, caryophyllene] were emitted. As damage proceeded, high levels of other terpenes, all acyclic [e.g., (*E*)-β-ocimene, (*E*)-β-farnesene], were emitted in a pronounced diurnal fashion; maximal emissions occurred in the afternoon. These acyclic terpenes followed this diurnal pattern of emission, even after removal of the caterpillars, although emission was in somewhat smaller amounts. In contrast, the emission of cyclic terpenes almost ceased after the caterpillars were removed.**

Plant odors have long been of interest because they attract phytophagous insects. Additionally, a rapidly growing body of evidence has implicated plant odors in the attraction of species that prey on or parasitize herbivorous insect pests (1). In the cases so far reported, plants that were nearly odorless before feeding damage emitted large quantities of volatile compounds in a delayed response to herbivore feeding (2). These induced odors have been shown to be powerful attractants for parasitic Hymenoptera (2) and predatory mites (3). However, this mechanism of self-defense by plants has been explored in only a few species.

In recent studies of both corn, *Zea mays* (2), and cotton, *Gossypium hirsutum* (4), seedlings we found that plants release a significantly greater number of compounds and larger amounts of total volatile compounds after overnight feeding damage by insects than when they have been freshly damaged by the insects. However, there was no previous indication that plants respond to herbivore damage with a diurnal rhythm of volatile compound emission, although a number of investigations have demonstrated the rhythmic nature of volatile compound release from flowers (5, 6). In many cases it appears that the release of volatile compounds by flowers is timed to coincide with the period of greatest activity of their pollinators. For example, Heath *et al.* (7) found that emission of floral odor by night-blooming jessamine peaked in the first 2 hr of the scotophase, coincident with the period of maximum feeding activity of the nocturnal moths that pollinate the jessamine. If plants are signaling to natural enemies of their herbivore attackers, it would seem likely that they would emit volatile compounds in greater quantities during the period when the natural enemies are actively hunting for hosts or prey. Thus, we investigated the release of volatile compounds from beet armyworm-damaged cotton leaves throughout three consecutive photoperiods to detect any diurnal variations in volatile compound emissions that might occur.

MATERIALS AND METHODS

Cotton plants, cv. Delta Pineland 90, were greenhouse grown in 16-cm-diameter pots in top soil/vermiculite/peat moss, 60:20:20, until ≈1.5 mo old. The plants were 25–30 cm tall and had not set flower buds. Beet armyworms (*Spodoptera exigua* Hübner) were reared by the method of King and Leppla (8).

The collection apparatus for volatile compounds used a push/pull technique and has been described in detail by Heath and Manukian (9). Briefly, the apparatus consisted of an 8-cm-diameter by 34-cm-tall glass sleeve that contained the plant. Air entered the top of the sleeve through multiple layers of an activated charcoal-infused fabric and passed down over the plant at a rate of 5 liters·min^{-1}. At the bottom of the sleeve a split plate with a hole in the center closed loosely around the stem of the plant like a guillotine to prevent air from flowing back into the sleeve from outside. The bottom of the sleeve was connected to a base that accommodated eight filter traps, arranged concentrically, for volatile compound collection. During collection, air was pulled through one of the traps at a rate of 1 liter·min^{-1}, and the remaining 4 liters·min^{-1} were vented out the bottom of the apparatus. Each trap contained 50 mg of Super Q adsorbent (Alltech Associates) and was conditioned before use by rinsing with 2 ml of CH_2Cl_2. Timing of the volatile compound collections was accomplished by computer-automated switching valves operated under the control of software developed in this laboratory (9, 10).

Volatile compounds were collected during May and June, 1993, in Gainesville, Florida. In the first series of experiments

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second-instar caterpillars were isolated at 0800 hr and starved until placed on the plants at 1400 hr. Five larvae were placed on each plant in the collection apparatus and the hole at the bottom of the chamber around the plant stem was loosely plugged with cotton to prevent larval escape. Volatile compound collections were begun at 1500 hr, were of 3-hr duration in each trap, and lasted for 60 hr, during which time caterpillars were allowed to feed continuously. All experiments were replicated five times, and the resulting data were subjected to ANOVA, and means were compared by a least-significant-difference test (11). An equal number of head-space collections from undamaged cotton plants were done for comparison.

Volatile compounds were eluted from the traps with 150 μl of CH_2Cl_2, 600 ng each of *n*-octane and nonyl acetate were added as internal standards, and 2-μl aliquots of all samples were analyzed by gas chromatography (GC) on a 50-m × 0.25 mm (i.d.) fused silica column with a 0.25-μm-thick bonded methyl silicone stationary phase (Quadrex, New Haven, CT). Injections were made in the splitless mode for 30 sec, and the gas chromatograph was operated under the following conditions: injector 220°C, detector 240°C, column oven 50°C for 3 min, then programmed at 5°C·min^{-1} to 190°C, He carrier gas linear flow velocity 21 cm·sec^{-1}. Selected samples were also analyzed on a 50 m × 0.25 mm (i.d.) fused silica column with a 0.25-μm-thick bonded cyanopropyl silicone (CPS-1; Quadrex) stationary phase operated under the following conditions: 30-sec splitless delay after injection, oven temperature 60°C for 1 min and then programmed at 5°C·min to 180°C, injector temperature 220°C, detector temperature 260°C, He carrier gas linear flow velocity 19 cm·sec^{-1}. For mass spectral analyses, 1-μl samples were introduced via a methyl silicone column, operated as before, into a Finnigan-MAT ITS40 (ion trap) mass spectrometer operated in the electron impact mode. Constituents of the plant volatile emission were identified by comparison of mass spectra with spectra in the Environmental Protection Agency–National Institutes of Health data base and spectra obtained of authentic compounds, and by comparison of GC retention times with those of authentic standards on both capillary columns.

RESULTS AND DISCUSSION

Representative chromatograms of head-space volatile compounds from beet armyworm-damaged cotton plants are shown in Fig. 1. Early stages of damage were characterized by high levels of lipoxygenase-derived volatile compounds [e.g., (*Z*)-3-hexenal, (*Z*)-3-hexenyl acetate] and a number of terpene hydrocarbons such as α-pinene, myrcene, and caryophyllene (Fig. 1*A*). As damage progressed, however, a number of other terpenes, which in early stages of damage had been released only in relatively small amounts, started to increase (Fig. 1*B*). Thus, whereas in early stages of damage a mixture of cyclic (e.g., α-pinene, caryophyllene) and acyclic terpenes (myrcene) predominated, later different terpenes, which appear to have been induced by beet armyworm-feeding damage, prevailed. These latter terpenes were all acyclic and were identified as (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*E,E*)-α-farnesene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

The increase in emission of the inducible terpenes began on the morning after the start of feeding damage and peaked during the afternoon. For example, immediately after the start of feeding during the 1500- to 1800-hr collection period, the cotton plants emitted high levels of α-pinene (>5 μg over the 3-hr collection period) but only small amounts of (*E*)-β-ocimene and (*E*)-4,8-dimethyl-1,3,7-nonatriene (Fig. 2). However, the next day levels of the latter terpenes started to rise rapidly, and in the peak hours of emission (i.e., from 1200 to 1500 hr and from 1500 to 1800 hr) production of (*E*)-β-ocimene and (*E*)-4,8-dimethyl-1,3,7-nonatriene averaged over 20 μg and 13 μg, respectively. These values were >10-fold increases in emission. Similar changes occurred in

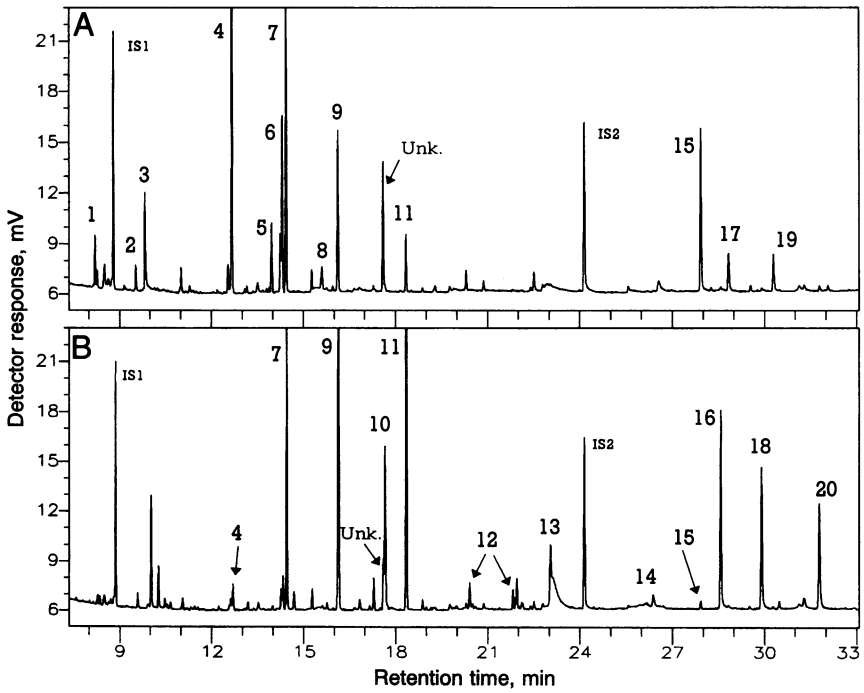

FIG. 1. Typical chromatograms of volatile compounds emitted from 1500 to 1800 hr by cotton plants subjected to beet armyworm-feeding damage. (*A*) First day of feeding damage. (*B*) Third day of feeding damage. Peak identities: 1, (*Z*)-3-hexenal; 2, (*E*)-2-hexenal; 3, (*Z*)-3-hexenyl acetate; 4, α-pinene; 5, β-pinene; 6, myrcene; 7, (*Z*)-3-hexenyl acetate; 8, limonene; 9, (*E*)-β-ocimene; 10, linalool; 11, (*E*)-4,8-dimethyl-1,3,7-nonatriene; 12, isomeric hexenyl butyrates; 13, indole; 14, (*Z*)-jasmone; 15, caryophyllene; 16, (*E*)-β-farnesene; 17, α-humulene; 18, (*E,E*)-α-farnesene; 19, γ-bisabolene; 20, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. IS1, *n*-octane; IS2, nonyl acetate; Unk., identity unknown.

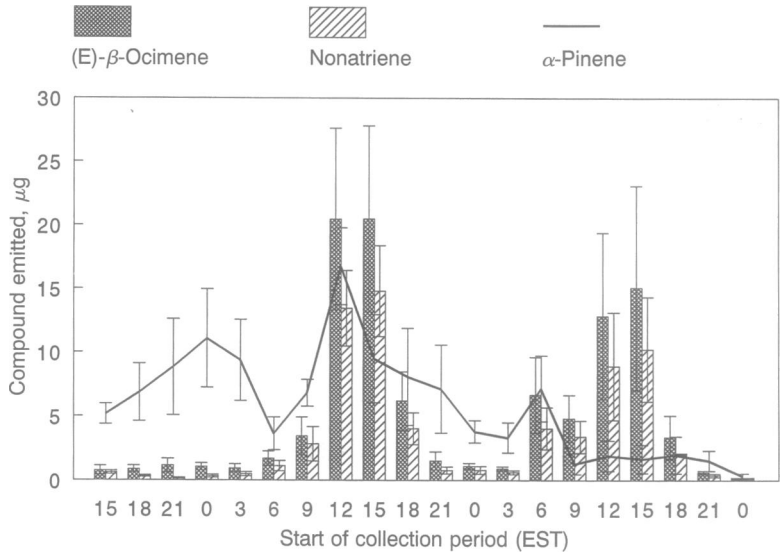

FIG. 2. Emission of α-pinene, (*E*)-β-ocimene, and (*E*)-4,8-dimethyl-1,3,7-nonatriene by cotton plants subjected to beet armyworm-feeding damage over the course of several days. Data represent the mean of five replications ± SE. EST, Eastern standard time.

the emission of the sesquiterpenes (Fig. 3), where in early stages of damage high levels of caryophyllene were emitted, whereas the following day high levels of (*E*)-β-farnesene and (*E,E*)-α-farnesene were released.

The emission of the inducible terpenoids exhibited distinctly diurnal periodicity. As illustrated in Figs. 2 and 3, emissions peaked strongly in the late afternoon on the second and third days with relatively low levels of emission at night. In contrast to the inducible terpenes, the emission of the other terpenes, such as α-pinene and caryophyllene, followed no distinct pattern. Rather, levels of these compounds slowly increased until they peaked on the second day of damage and then slowly declined thereafter.

Relatively low levels of volatile compounds were collected from undamaged plants, and the most abundant components were α-pinene, (*E*)-β-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Fig. 4). A diurnal pattern of periodicity was again noted for the latter three compounds. Although the possible effects of minor damage to the cotton plants from insects present in the greenhouse cannot be excluded, the levels detected from undamaged plants may suggest that insect-feeding damage amplifies an internal rhythm in cotton leaves. Beet armyworm larvae are nearly odorless (2) and thus can be excluded as a source of volatile compounds.

Because cotton plants accumulate large amounts of free monoterpenes and sesquiterpenes in specialized structures (12), the lack of a pattern of emission for compounds such as caryophyllene is probably due to their release by simple breakage of glands as a consequence of caterpillar feeding. Also, lipoxygenase-derived compounds, arising from the hydroperoxidation of fatty acids containing a *cis,cis*-1,4-pentadiene moiety, are known to be released as a result of mechanical damage (13). Therefore, the emission of such compounds should decline rapidly upon the removal of the caterpillars. To test this hypothesis, the caterpillars were removed at 0850 hr on the second day of feeding damage. As shown in Fig. 5, the levels of α-pinene and (*Z*)-3-hexenal rapidly declined after the removal of the caterpillars, whereas the emission of (*E*)-β-ocimene still peaked in the late afternoon, although at lower levels than when the caterpillars were allowed to remain on the plants.

The pronounced changes that occurred in volatile emissions during the three afternoons that the plants were mon-

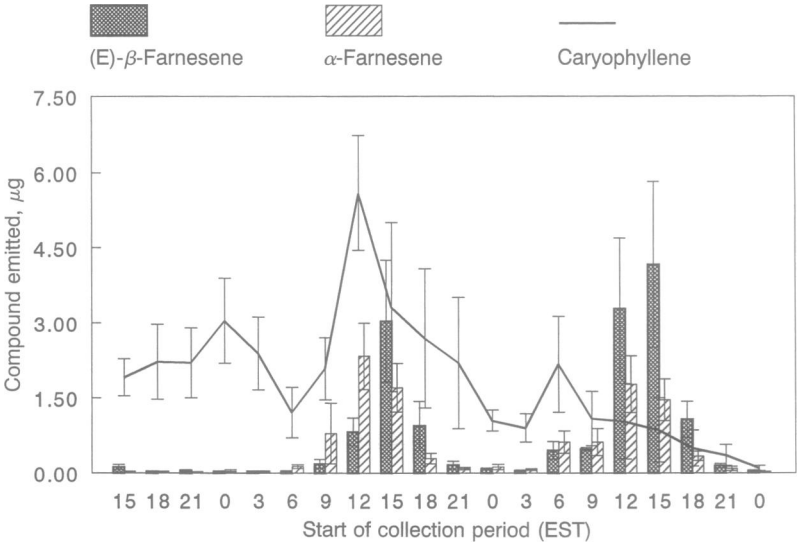

FIG. 3. Emission of caryophyllene, (*E*)-β-farnesene, and (*E,E*)-α-farnesene by cotton plants subjected to beet armyworm-feeding damage over the course of several days. Data represent the mean of five replications ± SE.

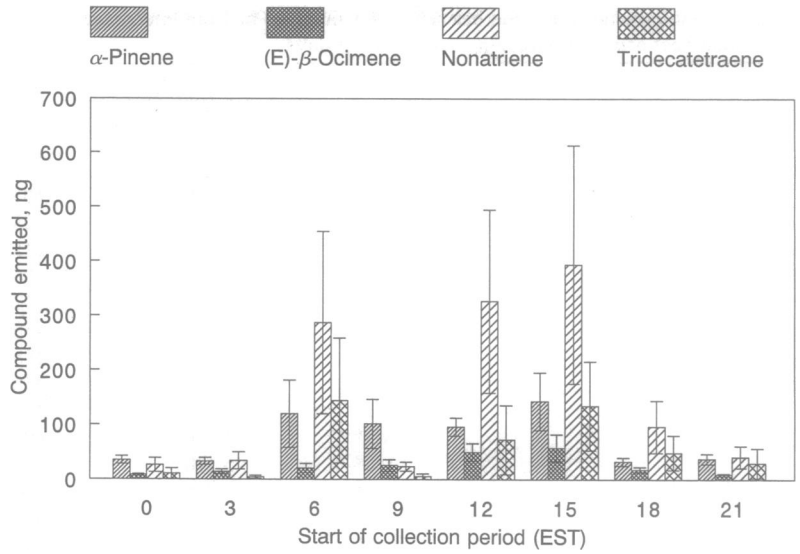

FIG. 4. Emission of α-pinene, (*E*)-β-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene from undamaged cotton plants. Data represent the mean of five replications ± SE.

itored are summarized in Table 1, along with levels emitted by undamaged plants during the same time period. On the first day of feeding damage, from 1500 to 1800 hr, total production of identified components averaged ≈6000 ng·hr^{-1}. On the second day during the same time period, this production had increased to >35,000 ng·hr^{-1}. The striking increase in the levels of (*Z*)-3-hexenyl acetate that occurred warrants particular mention; during the same sampling period the levels of this compound had increased ≈20-fold from the first to the second day of feeding damage. Unlike the changes observed for the inducible terpenes, however, this compound declined after the second day of damage and did not conspicuously increase again.

The mechanism whereby terpenoid release is induced by the caterpillar-feeding damage is unknown. It is possible that release from glycosidically bound forms is involved (14). The terpene hydrocarbons could then arise via acid-catalyzed dehydration of parent alcohols (15). Alternatively, a group of terpene synthases could be inducible by the feeding damage. Mechanical damage has been shown to induce terpene cyclase activity in *Abies grandis* (16), although in this species the products produced by inducible cyclase activity did not differ greatly from those of constitutive activity.

The release of terpenoids by cotton differs from that found previously for corn (2). Whereas cotton begins to release several terpenoids as soon as damage occurs, corn appears to have no free terpenoids available for immediate release. Terpenoids are released by corn plants only several hours after insect herbivore damage begins. This difference may be a reflection of two distinct strategies that the plants use in defense against herbivores. As suggested by Coley *et al.* (17), fast-growing annuals like corn may initially invest most of their energy in growth rather than defense. In corn, energy appears to be committed to defense only when the plant is under attack. Slower growing perennials like cotton may invest in some constitutive defenses to be braced for and perhaps "discourage" possible herbivore attacks.

In addition to the constitutive terpenoids, our results show that cotton produces inducible terpenoids in response to herbivory. While these may add to the blend of toxic chemicals available to combat herbivores, they also may provide parasitoids and predators with reliable signals pointing out the location of potential hosts or prey. The odors induced by

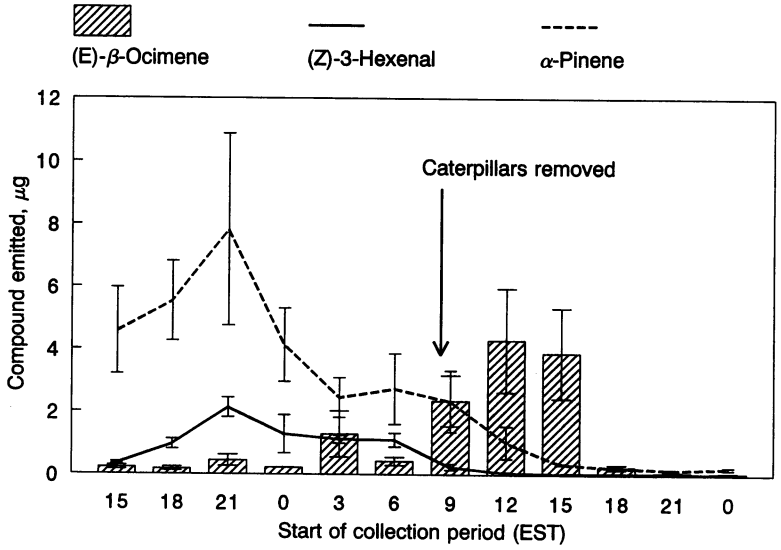

FIG. 5. Emission of (*Z*)-3-hexenal, α-pinene, and (*E*)-β-ocimene with caterpillars removed from the plant at 0850 hr Eastern standard time (EST). Data represent the mean of five replications ± SE.

Table 1. Composition of volatile blends collected between 1500 and 1800 hr from undamaged cotton plants and cotton plants subjected to continual beet armyworm-feeding damage for 3 days

Compound	ng of compound emitted			
	Undamaged plants	Beet armyworm-damaged plants		
		Day 1	Day 2	Day 3
(*Z*)-3-Hexenal	28*	799*†	1,603†	108*
(*E*)-2-Hexenal	21*	140*	618†	135*
(*Z*)-3-Hexenol	15*	610*†	1,515†	48*
(*Z*)-3-Hexenyl acetate	46*	1,325*	27,700†	3,710*
(*Z*)-3-Hexenyl butyrate	ND*	96*	812†	117*
(*E*)-2-Hexenyl butyrate	ND*	9*	458†	485†
(*Z*)-3-Hexenyl 2-methylbutyrate	ND*	6*	568†	200*
(*E*)-2-Hexenyl 2-methylbutyrate	ND*	3*	458†	329†
(*Z*)-Jasmone	ND*	181*†	482†	144*
Indole	ND*	3,330†	8,000†	4,160†
α-Pinene	143*	5,170*†	9,510†	1,680*
β-Pinene	30*	835*	1,520*	288*
Myrcene	77*	1,137*†	4,430†	1,470*†
Limonene	6*	505*†	737†	280*†
(*E*)-β-Ocimene	56*	772*	20,500†	15,100†
Linalool	10*	35*	2,510†	1,640†
(*E*)-4,8-Dimethyl-1,3,7-nonatriene	394*	616*	14,800†	10,200†
(*E,E*)-4,8,12-Trimethyl-1,3,7,11-trideca-tetraene	135*	103*	1,420†	1,530†
Caryophyllene	26*	1,910*†	3,310†	861*†
α-Humulene	11*	467*†	858†	271*†
γ-Bisabolene	10*	526*†	1,140†	270*†
(*E*)-β-Farnesene	5*	121*	3,030†	4,160†
(*E,E*)-α-Farnesene	2*	29*	1,700†	1,460†

ND, not detected.

Data represent the mean of five replications. Means within rows followed by the same symbol (* or †) are not significantly different ($P > 0.05$, least significant difference test).

the caterpillar-feeding damage have a distinctly floral character. In fact, it is remarkable how closely the rhythms of volatile emission noted here parallel those from some flowers (5, 6). In *Nicotiana sylvestris*, for example, while some compounds are emitted in a rhythmic fashion, others show no particular periodicity. It is interesting to note that many parasitic wasps exploit nectar as a food source (18). The few reports of studies of the diurnal patterns of parasitoid foraging in the field (19, 20) and our observations of the foraging behavior of the beet armyworm larval parasitoid *Cotesia marginiventris* in the laboratory indicate that these wasps forage during the middle to latter part of the photophase. Perhaps the induced release of large amounts of certain odors in damaged plants is similar to the periodic release of specific odors by flowers, in that it provides a clear and reliable signal, above the background of other plant odors, that attracts natural enemies of the herbivorous attackers.

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