

AN ELICITOR IN CATERPILLAR ORAL SECRETIONS THAT INDUCES CORN SEEDLINGS TO EMIT CHEMICAL SIGNALS ATTRACTIVE TO PARASITIC WASPS

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Abstract—Regurgitate of corn-fed beet armyworm (BAW) caterpillars, *Spodoptera exigua*, when applied to damaged sites of corn (*Zea mays*) seedlings, causes the release of relatively large amounts of terpenes by the seedlings several hours later. This plant response could be induced by merely placing the cut stem of seedlings in a solution of BAW regurgitate for 12 hr, a response that could not be induced by placing seedlings in water only. Regurgitate of BAW fed various diets, including a minimal diet of filter paper, were all active. However, seedlings placed in corn leaf juice, BAW hemolymph, or BAW feces extract released significantly smaller amounts of terpenes than did seedlings placed in BAW regurgitate. These results indicate that the active components are present in relatively large concentrations in regurgitate and that they are not related to the food source. Furthermore, regurgitate from several other species of caterpillars (*Spodoptera frugiperda*, *Helicoverpa zea*, *Trichoplusia ni*, and *Anticarsia gemmatilis*) as well as from the grasshopper *Schistocerca americana* induced the release of significant amounts of terpenes in corn seedlings. The release of these volatiles, therefore, appears to be a general response to attack by phytophagous insects. The terpene-releasing corn seedlings were highly attractive to the generalist parasitoid *Cotesia marginiventris* and to the specialized parasitoid *Microplitis croceipes*. This study confirms a systemic herbivore-elicited release of terpenes in corn. It is pro-

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posed that such chemicals serve multifunctional purposes that directly and indirectly protect plants against herbivorous arthropods and pathogens.

Key Words—Elicitor, corn, *Zea mays*, caterpillar regurgitate, plant volatiles, synomones, semiochemicals, parasitoids, *Cotesia marginiventris*, *Microplitis croceipes*, host searching.

INTRODUCTION

Plant odors play an important role in the foraging behavior of predators and parasitoids that attack herbivorous insects (Vinson et al., 1987; Nordlund et al., 1988; Williams et al., 1988; Tumlinson et al., 1992). In fact, previous studies show that plants under attack by herbivores may actually initiate the release of chemical signals that indicate the presence of prey or hosts to natural enemies. For instance, detailed studies on the tritrophic interaction between plants, spider mites, and predatory mites have revealed that spider mite-infested plants initiate the release of several terpenes and terpenoids that are exploited by predatory mites to locate their prey, the herbivorous spider mites (Dicke and Sabelis, 1988; Dicke et al., 1990a,b). Similarly, feeding damage caused by caterpillars induces corn seedlings to release relatively large amounts of terpenes and terpenoids that are attractive to the generalist parasitoid *Cotesia marginiventris* (Turlings et al., 1990, 1991b). Caterpillar-induced emissions of volatiles by plants are not limited to the damaged sites but occur throughout the plant, even in undamaged leaves (Turlings and Tumlinson, 1992). Such a systemic response is also indicated in spider mite-infested plants by the fact that predatory mites are attracted to uninfested leaves of mite-infested plants (Dicke et al., 1990b). Thus, infested plants provide natural enemies of herbivorous arthropods with chemical signals that guide them to the vicinity of potential hosts or prey.

The plant responses induced by mites or caterpillars cannot be mimicked with artificial damage (Dicke et al., 1990a; Turlings et al., 1990). Corn seedlings release only minor amounts of terpenes in response to artificial damage. However, when caterpillar regurgitate is applied to artificially damaged sites, corn seedlings released terpenoids in similar amounts as seedlings that had been damaged by caterpillars (Turlings et al., 1990). The specificity of the response indicates the presence of a factor in caterpillar regurgitate that causes plants to emit chemical signals. In the current study, we developed a bioassay in which terpene release could be induced in corn seedlings severed at the stem and placed in dilutions of caterpillar regurgitate. This easily standardized bioassay, whereby surface damage to the leaves was avoided, was used to test various alternative sources for activity. Additional bioassays with different caterpillar diets, different caterpillar species, and a grasshopper species were used to further characterize the active component(s) in the regurgitate. Treated seedlings also were

tested for their attractiveness to *Cotesia marginiventris*, a parasitoid that attacks a wide variety of lepidopterous caterpillars and *Microplitis croceipes*, which attacks *Heliothis* and *Helicoverpa* species only.

METHODS AND MATERIALS

Insects. All caterpillars were obtained from the USDA rearing facilities in Gainesville, Florida. They were reared according to the procedure described by King and Leppla (1984). *Schistocerca americana* grasshoppers were obtained from a colony maintained by Dr. J.L. Capinera at the Entomology Department at the University of Florida.

Cocoons of *Cotesia marginiventris* and *Microplitis croceipes* were obtained from colonies maintained at the USDA-ARS, Insect Biology and Population Management Research Laboratory, Tifton, Georgia. Both wasps were reared according to the procedure described by Lewis and Burton (1970) and held as described by Turlings et al. (1989). Flight-tunnel tests were conducted with 3- to 5-day-old mated females 4-8 hr into the photophase.

Corn. Corn (*Zea mays* L., var. Ioana sweet corn) was grown in metal trays (9 × 35 × 50 cm) in a greenhouse, approximately 60 seeds per tray. Seeds were planted in a 50:50 mixture of moist vermiculite and potting soil. Natural light was supplemented with 400-W high-pressure sodium lamps placed 1 m above the trays. Experiments were conducted with 8- to 10-day-old seedlings that carried three leaves.

Collection of Regurgitate. One day before their regurgitate was collected, 7- to 9-day-old caterpillars were placed on a specific diet (corn leaves unless stated otherwise). Regurgitation was induced by holding the caterpillars with a pair of light-weight forceps and gently pinching the head region with another pair. The regurgitate was collected by holding the caterpillar over a 100- μ l pipet that inserted into a 3-ml vial through a rubber septum, and attached to low vacuum via another pipet. This allowed the regurgitate to slowly drip into the vial through the pipet. Grasshopper regurgitate was collected in a similar fashion from hand-held insects. Depending to some extent on their diet, 10-25 μ l regurgitate could be collected from each caterpillar, while the grasshoppers produced 20-40 μ l. All regurgitate was centrifuged for 10 min at 11,750g and the supernatant was filtered through a 0.22- μ m sterile Millipore (Millex-GV) filter to remove bacteria.

Alternative Treatment Materials. To determine the specificity of the active component(s) in the caterpillar regurgitate, several arbitrarily chosen alternative sources were tested. A total of 1 ml of feces from BAW larvae fed on corn was collected, diluted in 5 ml of distilled water, and vigorously shaken for several minutes. The solution was centrifuged at 14,000 rpm for 30 min, the supernatant

filtered through a 0.22- μm Millipore filter, lyophilized, and rediluted with distilled water to a total volume of 1 ml. BAW hemolymph was obtained by piercing the larvae with a needle and gently squeezing the hemolymph out. Hemolymph was centrifuged and the supernatant filtered through a 0.22- μm filter. Corn juice was obtained from corn seedlings by first pulverizing them with a mortar and pestle. The seedlings were then wrapped in aluminum foil and squeezed so that additional fluid would drip from an open end in the foil back into the mortar. The corn juice was also centrifuged and the supernatant filtered.

Treatment of Seedlings. Seedlings were placed in 1-ml vials that contained either 500 μl of distilled water only (control) or 50 μl of treatment material (in most cases caterpillar regurgitate) diluted in 450 μl distilled water. Six treatments (including control) were tested on a given day with three seedlings per treatment. Experiments were replicated six times. The seedlings were cut from the trays in the greenhouse and placed in vials at 9–11 PM. The following day at 9–10 AM, they were removed from the vials, the submerged part of the stem was cut off, and the severed end was wrapped in wet cotton wool.

Collection and Analysis of Volatiles. The volatile collection system used was the same as previously described by Turlings et al. (1991b). Each group of three seedlings that underwent the same treatment was placed in a glass chamber (3 cm ID \times 15 cm long). Using a push-pull technique (compressed air and vacuum), humidified clean air (500 ml/min) was passed through the chamber and through an adsorbent (25 mg Super Q) that trapped the volatiles emitted by the seedlings. Six collection systems were used in parallel and collections lasted 2 hr.

After collection, the traps were rinsed with 150 μl methylene chloride and an internal standard (600 ng of nonyl acetate in 30 μl methylene chloride) was added. Two microliters of each sample was analyzed on a 50-m \times 0.25-mm-ID bonded methyl silicone (007) (0.25- μm -thick film) capillary gas chromatography column combined with a 10-m \times 0.25-mm deactivated retention gap. The Hewlett-Packard model 5890 gas chromatograph (GC) was equipped with an on-column injector system and a flame ionization detector and helium (19 cm/sec) was used as a carrier gas. Following injection, column temperature was maintained at 50°C for 3 min and then programmed at 7°C/min to 190°C. A PE Nelson (Cupertino, California) data collection system was used, and the eight most predominant compounds were quantified using Turbochrome 3.1 software. The total amounts of these eight compounds were used to compare induced releases among different treatments.

Flight-Tunnel Bioassays. Attractiveness of seedlings to the parasitoids *C. marginiventris* and *M. croceipes* was tested in the Plexiglas flight tunnel described by Turlings et al. (1991a). Conditions in the tunnel were: 15 cm/min airflow;

55–70% relative humidity; 27.5–29°C; and approximately 500 lux illumination. For each experiment, groups of three seedlings that had undergone the same treatment were placed in one vial. Two vials with seedlings were placed 20 cm apart on a 40-cm-high stand at the upwind end of the tunnel. Each vial contained seedlings that had undergone different treatments: they were seedlings that had been standing either in distilled water only, diluted BAW regurgitate, or grasshopper regurgitate. Female wasps were released 80 cm downwind from the seedlings. Before release the wasps had been given a 20-sec contact experience with a complex of corn seedlings fed upon by BAW larvae (in the case of *C. marginiventris*) or *Helicoverpa zea* larvae (in the case of *M. croceipes*). Such experiences greatly increase their responses to host-related odors (Drost et al., 1986; Eller et al., 1988; Turlings et al., 1989). On a given day, six wasps of one species were tested to each of the three possible combinations of seedlings; this was repeated on six different days ($N = 36$). We recorded the numbers of females landing on each group of seedlings.

RESULTS

Induced Release of Volatiles. The amounts of volatiles emitted by seedlings that had been standing in a 10-fold dilution of BAW regurgitate differed dramatically compared with the volatiles emitted by seedlings that spent the same amount of time (12 hr) in water only (Figure 1). The identity of the components, mostly terpenes, had been determined in a previous study (Turlings et al., 1991b).

Alternative Sources. The effects of BAW regurgitate on the volatiles released by corn seedlings were compared with the releases caused by BAW feces, BAW hemolymph, corn juice, and regurgitate from another herbivorous insect, the grasshopper *Schistocerca americana*. After having been placed in vials with 10-fold dilutions of the various treatments, all seedlings released volatiles in significantly larger total amounts than did control seedlings (Figure 2). The regurgitate treatments, however, resulted in significantly larger total amounts of terpenoids; the grasshopper regurgitate was particularly active. Of the five treatments, BAW feces and corn juice were the least active.

Different Diets. Regurgitate from BAW larvae fed on a variety of diets was tested to determine if diet influences activity. For this purpose regurgitate was collected from BAW larvae that, for one day, had been fed corn leaves, cotton leaves, soy leaves, artificial diet (a pinto bean-based diet; King and Leppla, 1984), or sucrose water-sprinkled filter paper (Whatman 1). While regurgitate from all diets induced the release of terpenes in corn seedlings, regurgitate from caterpillars fed on soy leaves was most active and regurgitate from cotton- and filter paper-fed larvae was the least active (Figure 3).

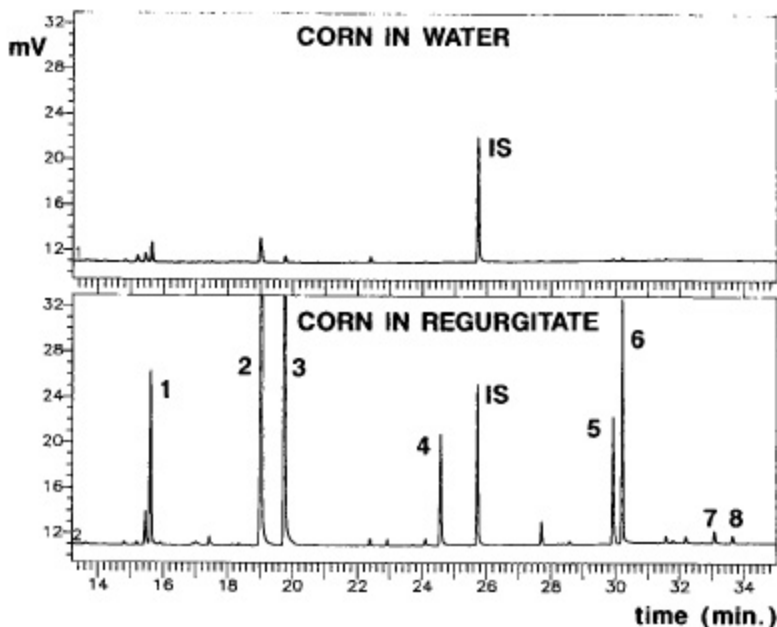


FIG. 1. Comparison of chromatographic profiles of volatiles released by corn seedlings that were placed for 12 hr in vials with distilled water or vials with a 10-fold dilution of BAW regurgitate. The identities of the various compounds have been determined previously (Turlings et al., 1991b); they are: 1, (*Z*)-3-hexen-1-yl acetate; 2, linalool; 3, (*3E*)-4,8-dimethyl-1,3,7-nonatriene; 4, indole; 5, α -*trans*-bergamotene; 6, (*E*)- β -farnesene; 7, (*E*)-nerolidol; and 8, (*3E*, *7E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. As an internal standard (IS) we used nonyl acetate (600 ng).

Different Caterpillar Species. To learn more about the specificity of regurgitate activity, regurgitate from five species of caterpillars was collected and tested. In addition to regurgitate from BAW, we collected regurgitate from *Spodoptera frugiperda* (fall armyworm = FAW), *H. zea* (corn earworm = CEW), *Trichoplusia ni* (cabbage looper = CL), and *Anticarsia gemmatilis* (velvetbean caterpillar = VBC). All caterpillars had been feeding on artificial diet. The regurgitate of each species caused corn seedlings to initiate the release of terpenoids (Figure 4). Regurgitate from BAW, CEW, and CL was consistently more active than regurgitate from FAW and VBC.

In all cases, treatments induced the release of the same blend of eight compounds. Differences were only consistently observed in the total amounts released, but not in the relative ratios of the compounds.

Parasitoid Responses. In a flight tunnel, female wasps were given a choice between combinations of corn seedlings that had been standing in either distilled

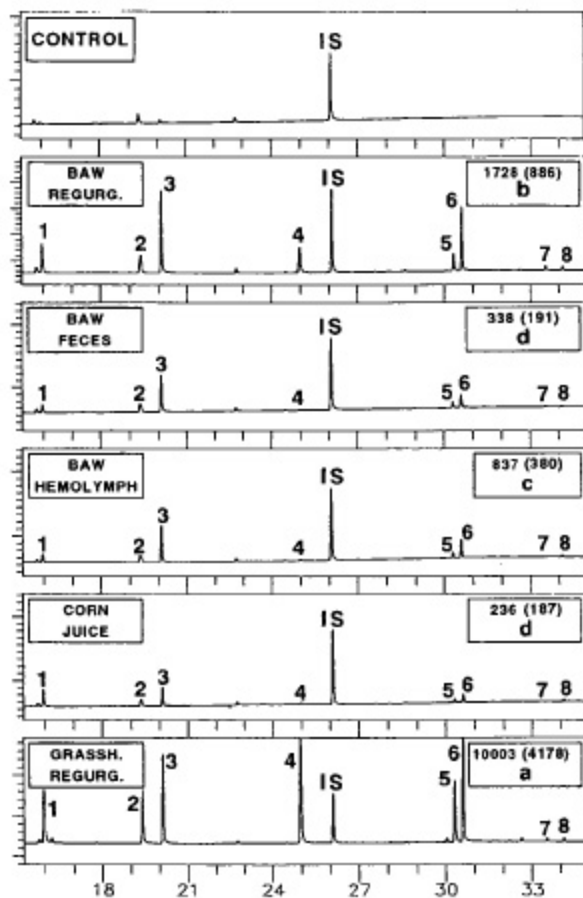


FIG. 2. Comparison of volatiles released by corn seedlings that were placed in distilled water (CONTROL), BAW regurgitate, BAW feces, BAW hemolymph, corn juice, and grasshopper regurgitate. Identities of peaks are given in Figure 1. Values in the right-hand corners are the mean ($N = 6$) total amounts (ng/3 seedlings/2 hr) of the eight compounds released and their standard deviations. Means with different letters are significantly different (paired t test, $P < 0.05$).

water, diluted BAW regurgitate, or diluted grasshopper regurgitate. Both *C. marginiventris* and *M. croceipes* showed a clear preference for seedlings treated with regurgitate (Figure 5). Neither wasp showed a significant preference when given a choice between corn seedlings treated with either caterpillar regurgitate or grasshopper regurgitate, although *C. marginiventris* may have shown a tendency to fly more often to grasshopper regurgitate-treated corn.

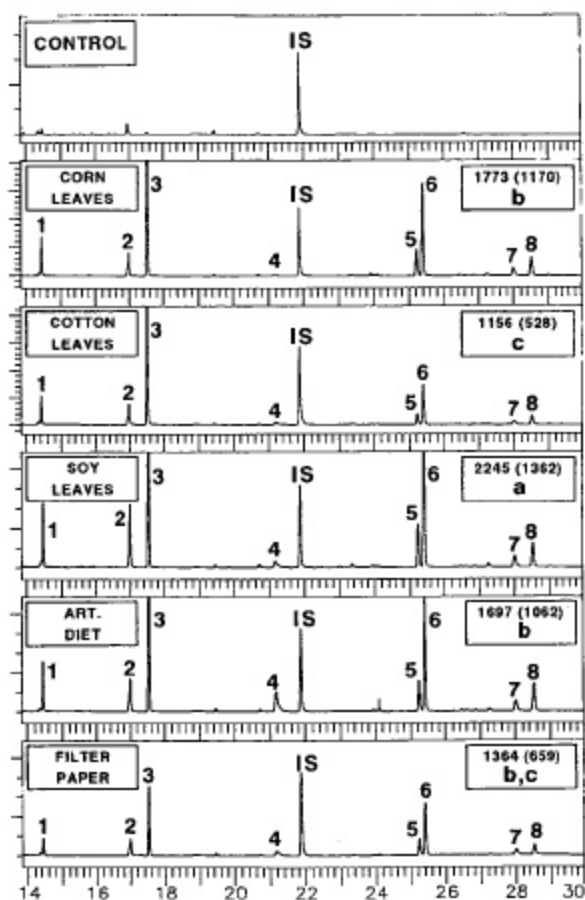


FIG. 3. Comparison of volatiles released by corn seedlings that were placed in regurgitate from BAW caterpillars that had been fed different diets. Identities of peaks are given in Figure 1. Values in the right-hand corners are the mean ($N = 6$) total amounts (ng/3 seedlings/2 hr) of the eight compounds released and their standard deviations. Means with different letters are significantly different (paired t test, $P < 0.05$).

DISCUSSION

Injury inflicted by herbivores can induce the production of specific chemicals in various plants. These chemicals may serve to heal the wounds, as antibiotics to prevent secondary infections by pathogens, or as toxins or repellents against herbivores (Levin, 1976; Kogan and Paxton, 1983; Rhoades, 1979, 1985; Schultz, 1988; Tallamy and Raupp, 1991). More recent work (Dicke and

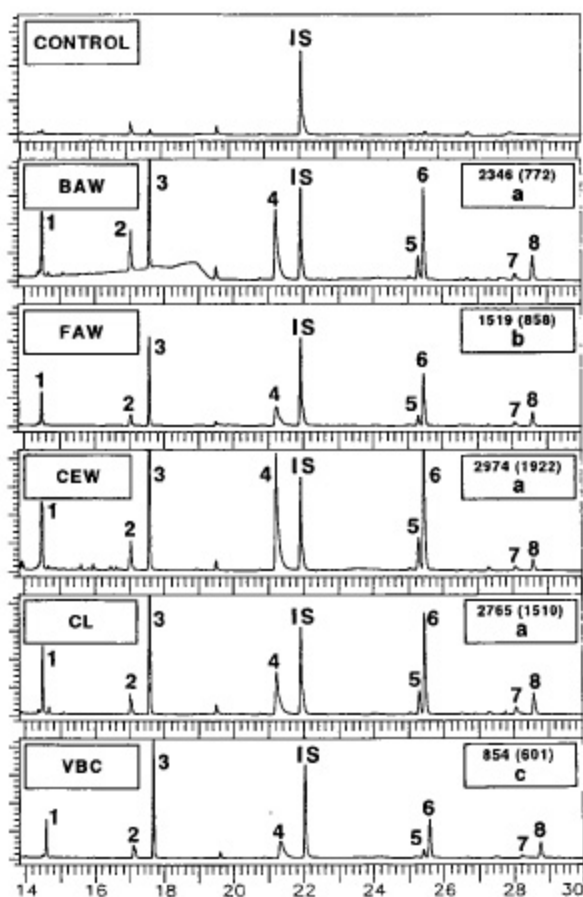


FIG. 4. Comparison of volatiles released by corn seedlings that were placed in regurgitate from different caterpillar species (BAW = beet armyworm; FAW = fall armyworm; CEW = corn earworm; CL = cabbage looper; VBC = velvetbean caterpillar). Identities of peaks are given in Figure 1. Values in the right-hand corners are the mean ($N = 6$) total amounts (ng/3 seedlings/2 hr) of the eight compounds released and their standard deviations. Means with different letters are significantly different (paired t test, $P < 0.05$).

Sabelis, 1988; Dicke et al., 1990a,b; Turlings et al., 1990, 1991b; Turlings and Tumlinson, 1991) also shows that plants, in response to damage caused by herbivorous arthropods, may actively release volatile chemicals that attract natural enemies of the herbivores.

The generalist parasitoid *C. marginiventris* (Turlings et al., 1990, 1991a,b) and the specialized parasitoid *M. croceipes* (Elzen et al., 1987; Drost et al.,

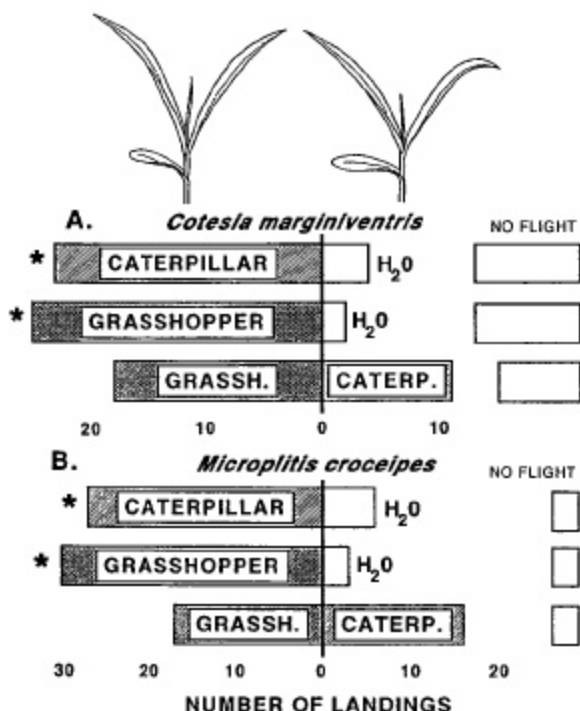


FIG. 5. Number of landings by *Cotesia marginiventris* (A) and *Microplitis croceipes* (B) females on corn seedlings during two-choice tests in a flight tunnel. Seedlings had spent 12 hr in either distilled water, BAW (caterpillar) regurgitate, or grasshopper regurgitate. Asterisks indicate statistically significant preferences for indicated treatments (chi-square, $P < 0.05$). The "No Flight" bars represent those wasps that did not land on a plant.

1988; Eller et al., 1988; McCall et al., 1993) both use plant-released volatiles in host habitat location. Here we show that the release of volatiles that strongly attract these parasitoids can be induced in corn with an elicitor in the regurgitate of herbivorous insects, without actually damaging the surface of the leaves. This confirms previous findings that the plant response is systemic and that the volatile releases are not limited to injured sites, but occur throughout the plant (see also Turlings and Tumlinson, 1992). However, in order for a plant to respond, the elicitor does need to enter the plant (in this case through the severed stem); rubbing regurgitate over intact leaves is not enough to cause a response (Turlings et al., 1990).

The Elicitor and Its Mode of Action. The exact source of the elicitor(s) was not pinpointed in this study, but since activity did not depend on the diet of the caterpillars (even regurgitate of caterpillars fed on filter paper was active), the

compound(s) are likely to be insect produced and are perhaps excreted from glands near the mouth parts. Furthermore, the results show that eliciting factors are common to lepidopterous larvae and may occur in a variety of herbivorous insects. Since they occur not only in regurgitate of caterpillars but also in small quantities in other natural sources and in very large quantities in grasshopper regurgitate, it is likely that they are common natural compounds. We propose that these factors indicate to the plant that it is being attacked by phytophagous organisms, and that, as hypothesized previously (Turlings et al., 1990; Turlings and Tumlinson, 1991), the plant response is a defensive reaction primarily directed at invading herbivores and/or pathogens. Lin et al. (1990) found that such induced resistance in soybean could be greatly enhanced by applying soybean looper regurgitate to damaged plant tissue. Detling and Dyer (1981) showed that grasshopper regurgitate reduces growth in blue grama grass, which also might indicate that energy investments are shifted towards defenses.

The elicitor appears to be taken up by the plant and induces the synthesis and/or release of specific terpenes. Many questions remain concerning the mode of action of the elicitor and the biochemical pathway(s) it may trigger. It may be that the elicitor is transported throughout the plant, or that it simply induces the production and transportation of other products at the point of entry. It is also still unknown whether actual synthesis takes place or whether the terpenoids are mobilized from elsewhere in the plant. Thompson et al. (1974) found only minor amounts of the terpenoids in the essential oil from healthy corn, suggesting that the plant response either involves synthesis of the volatiles or that they are bound in healthy plants and freed when the plant is attacked.

Functions of Plant-Released Volatiles. Terpenes and sesquiterpenes are clearly important for prey and host location by predators and parasitoids of phytophagous arthropods. However, the release of these chemicals is most likely a direct defense against herbivores and against pathogens that may invade the injured plants. Examples of the detrimental effect of terpenes and terpenoids on the development of insect larvae are ample (Mabry and Gill, 1979; Turlings and Tumlinson, 1991), and these chemicals are frequently implicated in an antibiotic role (Loomis and Croteau, 1980; Brooks and Watson, 1985). Yet the defensive roles of induced chemical changes in plants have recently been questioned (e.g., Fowler and Lawton, 1985; Myers and Williams, 1987; Coleman and Jones, 1991; Faeth, 1988, 1991). Many of the studies on plant defenses involve tree species that may largely rely on constitutive defenses rather than induced defenses and are mainly subjected to attacks by well-adapted species. This could explain the limited evidence for the detrimental effect of the induced chemical changes on individuals or communities of the phytophagous insects. Defensive strategies are different in many fast-growing annuals that often possess highly flexible defense expressions that are activated only when the plants are under attack (Coley et al., 1985).