Journal of Chemical Ecology, Vol. 28, No. 1, January 2002 (© 2002)

A NOVEL FUNCTION OF THE TRITERPENE SQUALENE IN A TRITROPHIC SYSTEM

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(Received March 30, 2001; accepted September 3, 2001)

Abstract—Changes in apple leaf chemistry after infestation by leafminers and their effect on both host location and host habitat location of the generalist parasitoid *Pholetesor bicolor* were investigated. Chemical analysis of leaf solvent extracts from healthy and leafminer-damaged leaves revealed that herbivory increased the amount of the triterpene squalene ($C_{30}H_{50}$), whereas quantities of all other identified compounds were similar in both plant treatments. To assess the response of parasitoids to host location cues, contact bioassays were conducted with naïve females. Results showed that parasitoids performed a characteristic ovipositional probing more often on the mine-damaged than on the healthy leaf. This behavior was triggered by a hexane extract of the mine-damaged leaf, but not by a healthy leaf extract. A synthetic mixture of the compounds identified in the extract triggered a similar response. A mixture devoid of squalene was not active, whereas squalene alone elicited the probing behavior. To assess the use of the identified compounds in habitat location, Y-tube olfactometer experiments were conducted with naïve and experienced females. Results showed that squalene is not involved in habitat location and has no priming effect on *P. bicolor*. While other triterpenes are known to mediate habitat location of parasitoids, this is the first report in which a plant triterpene is shown to mediate host location of a parasitoid. The biological and ecological functions of squalene on all three trophic levels are discussed.

Key Words—Squalene, semiochemicals, foraging behavior, leafminer parasitoid, apple plant, tritrophic.

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INTRODUCTION

Upon insect herbivory, plants generally activate biochemical defenses. These defenses may act directly against herbivores (reviewed by Karban and Baldwin 1997) and/or elicit chemical signals that may attract natural enemies of the herbivore (reviewed by Price 1997). Several plant compounds induced by herbivore feeding have been identified and are most often used by parasitoids during the initial stages of host selection, i.e., for habitat location before landing (Rutledge, 1996). It is relatively unknown how parasitoids use plant chemicals after landing on an infested plant. In fact later stages of host selection, including host location, host acceptance, and oviposition (*sensu* Vinson, 1984) are most often mediated by semiochemicals directly produced by the arthropod host (Vinson, 1991; Vet and Dicke, 1992). There seem to be exceptions where plant derived chemical cues mediate host location of parasitoids. This includes foraging of parasitoids for non-concealed (Horikoshi et al., 1997) as well as concealed hosts (Keller and Horne, 1993; Horikoshi et al., 1997; Mattiacci et al., 1999).

Endophytic hosts such as leafminers impose a particular challenge for natural enemies. Parasitoids searching on a leaf for concealed hosts do not have direct contact with the herbivore. This investigation deals with the chemical signals involved in host location of *Pholetesor bicolor* Ness (Hymenoptera: Braconidae). This endoparasitoid belongs to a large community of natural enemies that parasitize apple leafminers of the genus *Phyllonorycter* (Lepidoptera: Gracillariidae). In Hungarian orchards, *P. bicolor* is considered a primary parasitoid of apple leafminers (Balázs et al., 1996). Because *P. bicolor* is well synchronized with its host (Balázs, 1992), it can be considered a potential biological control agent of *Phyllonorycter* species that cause economic damage in several major apple growing regions of the world (Dorn et al., 1999).

In a recent study on host location of *P. bicolor*, we obtained results indicating an innate response of parasitoids to the herbivore-damaged leaf, but not to the larva or host-derived material (Dutton et al., 2000). The response could be elicited by exposing females to a synthetic mixture of *n*-alkanes and squalene, which were identified from the herbivore-damaged mine area. *n*-Alkanes are important chemicals in both habitat and host location for various parasitoid species (Rutledge, 1996). However, in this system it remains unclear whether these compounds are produced by the plant after leafminer herbivory, which specific compound(s) are used by the parasitoid for host location, and whether they are used by the parasitoid as chemical cues for habitat location.

Habitat location of *P. bicolor* involves olfactory cues emitted by infested leaves (Dorn et al., 1999). Given that this parasitoid has no direct contact with the host upon landing on a leaf, it is possible that similar compounds mediate both habitat and host location. Furthermore, as a generalist parasitoid, *P. bicolor* may benefit from a previous contact exposure to the leaf enclosing its endophytic host

for the subsequent location of leafminers in the same habitat. Indeed, experience is known to increase parasitoid responses towards certain stimuli (i.e., priming) or even generate new ones (i.e., associative learning). For example, previous contact exposure to innately recognized semiochemicals enhances the searching ability of parasitoids used for biological control in greenhouses and the field (Hare et al., 1997; Grasswitz, 1998). *P. bicolor* prefers volatiles emitted by infested over noninfested plants only after prior exposure to plants damaged by leafminers (Lengwiler et al., 1994; Dorn et al., 1999). Presently, the effect of exposure to innately recognized contact cues on the subsequent habitat location of this parasitoid is unknown.

This study was designed to: (1) investigate the chemical response of apple leaves to leafminer herbivory, (2) identify the substance(s) mediating host location of *P. bicolor,* (3) assess the role of apple leaf chemicals for both host and habitat location, and (4) determine the impact of experience on contact cues for the subsequent parasitoid habitat location.

METHODS AND MATERIALS

Plant and Insect Rearing

Two-month-old apple seedlings (*Malus sylvestris* cv. Golden Delicious, Rosaceae) were used for rearing of insects and for preparation of leaf chemical extracts. Plants were grown in climatic chambers at 25°C daylight and 17°C night, 70% relative humidity and 16 L : 8 D photoperiod.

Insects were collected in apple orchards in South Tyrol (northern Italy). Parasitoids were reared in individual cages on four to five apple seedlings heavily infested with sap-feeder leafminers. At pupation, leaves were removed from plants and introduced in a separate cage in which the parasitoids were allowed to emerge. We used three to six-day-old honey-fed female parasitoids for all bioassays. Host location bioassays were conducted with mated naïve females, whereas habitat location experiments were conducted with females that were mated and then given an experience (see details below).

Plant Response to Herbivory

Leaf extracts were obtained by soaking overnight 45-50 apple leaves with 100 mines (2 mines per leaf) in 200 ml hexane. These herbivore-infested apple leaves are referred to as mine-damaged leaves. The hexane extract was filtered through 10 g silica gel (Supelco) that had been previously conditioned with 50 ml hexane, and concentrated first with a rotary evaporator (32 ± 3°C) and then under a stream of nitrogen (purity 99.96%) to 10 μl. The same extraction procedure was applied to healthy leaves that were of similar age and had the same leaf area surface as those with mines. Leaf area was measured with an area meter instrument (Licor 300A).

Ten separate sample extracts of each treatment (mine-damaged and healthy) were analyzed with an HP 5890 Series II Plus gas chromatograph, equipped with a flame ionization detector (300°C) and a nonpolar HP-1 (Crosslink Methyl Silicone Gum) capillary column (30 m × 0.25 mm × 0.25-μm film thickness). The oven temperature program was held at 100°C for 2 min, then increased to 300°C at a rate of 6°C/min, and held at 300°C for 15 min. The split/splitless injector (250°C) was operated in splitless mode throughout the analyses. The carrier gas was helium (purity 99.96%) at a pressure of 108 kPa (constant pressure). Samples of 1 μl were injected. To evaluate quantitative differences among treatments for the individual compounds identified, peak areas were analyzed using a *t* test with the appropriate Bartlett's test for homogeneity and Kolmogorov-Smirnov test for normality (Sokal and Rohlf, 1981). To quantify the compounds found in mine-damaged leaves and healthy leaves, three additional extracts of each treatment were analyzed with an internal standard (100 ng octylbenzene/μl). The quantities of each compound were calculated based on GC peak areas relative to the internal standard. A response factor for each identified compound in relation to the internal standard was calculated. Identification of compounds by coinjection with authentic standards was performed with gas chromatography–mass spectrometry (GC-MS). Analyses were done using a HP 6890 Series GC connected to an HP 5973 electron-impact (EI) MS. The chromatographic conditions were kept as described above. The MS temperature parameters were: 230°C at the source, 150°C at the quadruple, and 280° at the interface. The masses were swept at 23–600 mass units at a rate of 2.6 scans/sec.

Parasitoid Response to Contact Chemical Cues for Host Location

All behavioral contact assays were performed in a single choice Petri dish (10 cm diam.) arena. Parasitoids were placed individually in glass vials (2 cm diam. × 4 cm high) and the open end of the vial was positioned over the plant cue sources or over an extract treated filter-paper (1.3 cm diam.). Females that were observed performing the characteristic thrusting of the ovipositor, from here on referred to as probing, on the filter-paper for more that 5 sec were considered to respond positively to the treatment. Observations were conducted under laboratory conditions at 23 ± 2°C, 50 ± 10% relative humidity and 1000 ± 500 lux. Each treatment was bioassayed simultaneously with a control, and for statistical analysis a χ^2 test was performed between treatments and controls.

Experiment 1. Response to Healthy and Mine-Damaged Leaf Material. In order to elucidate whether the source of active compounds eliciting ovipositional probing of *P. bicolor* is specific to herbivore damage, female parasitoids were offered the following cue sources: (1) a 1.2-cm^2 piece of healthy lower-leaf epidermis (healthy), (2) a 1.2-cm^2 piece of lower-leaf epidermis damaged by one larva (mine-damaged), from which the larva and its by-products were

carefully removed, or (3) an empty Petri dish (control). The leaf area offered corresponded to the size of a fully developed mine. This reference area was used throughout the work for quantifying the compounds extracted from leaves and for calculating the concentrations of the synthetic mixture used in subsequent experiments.

The behavioral parameters recorded were the time spent walking, standing, and probing on the cue source offered. The parameters were recorded over a 20-min period with The Observer computer software (Noldus, 1991). Each behavioral parameter was analyzed by Kruskal-Wallis ANOVA followed by pairwise multiple comparisons between treatments using the Student-Newman-Keuls method (Sokal and Rohlf, 1981).

Experiment 2. Response to Healthy and Mine-Damaged Leaf Extracts. In order to examine whether host location is elicited by plant chemical cues, solvent extracts from both healthy and mine-damaged leaves were tested. Leaf epidermis from each plant treatment was carefully removed with tweezers and soaked separately in hexane (purity 99.7%) at 4°C for 24hr. The extract concentration applied to the filter-paper was made so that 10 μl were equivalent to 1.2 cm^2 mine-damaged or healthy leaf. Hexane alone was applied to the filter paper as a control.

Experiment 3. Response to a Mixture of n-Alkanes and/or Squalene. A mixture of the synthetic *n*-alkanes *n*-C_{27}, *n*-C_{29}, *n*-C_{30}, *n*-C_{31}, *n*-C_{32}, *n*-C_{33}, and squalene ($C_{30}H_{50}$) (Fluka Chemie AG, purity $\geq$ 99.5%) at quantities equivalent to 1.2 cm^2 mine-damaged leaf (see Table 1) was tested against a control (hexane). A dose–response test was carried out with the same reference mixture using a dilution factor of 10. The mixture of *n*-alkanes without squalene was tested, and subsequently a dose–response test of squalene was performed.

Parasitoid Response to Host Location Cues for Habitat Location

Two Y-tube olfactometer bioassays were conducted to examine: (1) whether the identified chemical cues identified in mine-damaged leaves are used by *P. bicolor* as olfactory stimuli for habitat location, and (2) whether a previous contact exposure to innately recognize plant-derived cues enhance habitat location of *P. bicolor* through priming (Hare et al., 1997).

The olfactometer consisted of a glass Y-shaped tube. Odor sources were placed in glass chambers (27 cm long $\times$ 2.5 cm diam.) which directly fitted to each of the two Y-tube arms. Humidified air (300 ml/min) entering the odor source chamber was filtered through a charcoal filter. Parasitoids were individually introduced in the central arm of the olfactometer and given a maximum of 10 min to make a choice for one of the two olfactometer arms. A choice was recorded when the female had crossed a line marked 20 cm after the junction of the two arms. Responses of *P. bicolor* in the olfactometer were analyzed with a χ^2 test

with null hypothesis of a 50 : 50 distribution over the two odor sources. Females that did not respond during the 10-min observation were not included in the analysis. To avoid a bias in arm preference, odor sources were reversed after every third tested female. The environmental conditions in the bioassay room were 23 ± 2°C, 50 ± 5% relative humidity and 1200 ± 50 lux provided with artificial light.

Experiment 4. Squalene and n-Alkanes as Olfactory Stimuli. In the Y-tube olfactometer females were given the choice between the synthetic mixture of squalene and *n*-alkanes identified in the mine-damaged leaves and pure solvent as control (blank). The mixture of compounds was tested at a concentration equivalent to 1.2 cm^2 mine-damaged leaf surface and at three higher concentrations (dilution factor of 10). The compounds in hexane were applied to a Teflon septum (1.3 cm diam.) and placed in one arm of the Y-tube. A similar septum with hexane (blank) was placed in the other arm of the Y-tube.

Prior to being tested, wasps were given 25 ± 5 min contact experience on leafminer-infested plants. Preliminary experiments showed that naïve parasitoids are not attracted to mine-damaged plants. Experienced wasps were placed individually in vials for 30 min before they were tested.

Experiment 5. Squalene and n-Alkanes as Priming Stimuli. In the Y-tube olfactometer, females were given the choice between one infested apple leaf containing five mines (mined leaf) or clean air (blank). Wasps were given either no experience (naïve) or a 25 ± 5 min contact experience on one of the following treatments: (1) a leafminer infested plant, (2) the hexane extract of mine-damaged leaf, (3) the synthetic mixture of squalene and *n*-alkanes, or (4) a healthy plant. The contact experience with leafminer infested or healthy plants was provided as in the previous experiment (see above). Experience on the leaf extract or the synthetic mixture was provided by offering the solution in a filter-paper as in the host location bioassays.

RESULTS

Plant Response to Herbivory. Eight major compounds were identified by GC-MS in extracts from apple leaves infested with the leafminer *P. pomonella* and from healthy apple leaves (Table 1). Among them, only squalene ($C_{30}H_{50}$) was found in the mine-damaged leaves in larger quantities than in the healthy leaves (Student's *t* test, t value $= 2.6$; $df = 9$; $P = 0.03$). Only one minor peak was not identified, and all other compounds consisted of long-chain hydrocarbons (*n*-C_{27}, *n*-C_{29}, *n*-C_{30}, *n*-C_{31}, *n*-C_{32}, *n*-C_{33}).

Experiment 1. Response to Healthy and Mine-Damaged Leaf Material. The behavior of *P. bicolor* offered mine-damaged or healthy leaf material is depicted in Figure 1. Wasps spent more time walking, standing, and probing on the mine-damaged leaf ($P < 0.05$, Kruskal-Wallis ANOVA) than on the healthy leaf.

TABLE 1. COMPOUNDS EXTRACTED WITH HEXANE FROM *Phyllonorycter pomonella* HERBIVORE-DAMAGED AND HEALTHY APPLE LEAVES (*Malus sylvestris*)[a]

Compound	Leaf		
	Herbivore-damaged	Healthy	*P*
Heptacosane *n*-C_{27}	10.7 ± 0.8	8.2 ± 0.7	n.s
Unknown	6.3 ± 1.1	4.0 ± 0.9	n.s
Squalene $C_{30}H_{50}$	32.3 ± 13.1	12.2 ± 4.7	<0.05
Nonacosane *n*-C_{29}	91.3 ± 8.9	77.4 ± 11.3	n.s
Triacontane *n*-C_{30}	8.7 ± 1.2	7.3 ± 0.8	n.s
Hentriacontan *n*-C_{31}	251.6 ± 36.3	228.8 ± 18.8	n.s
Dotriacontane *n*-C_{32}	5.9 ± 0.9	5.6 ± 0.6	n.s
Tritriacontane *n*-C_{33}	34.0 ± 5.5	34.2 ± 1.7	n.s
Total	440.8 ± 67.6	377.8 ± 39.5	n.s

[a] [Average amount (ng $\pm$ SE) extracted from 1.2 cm^2 leaf surface (*t* test between treatments, $N = 10$)].

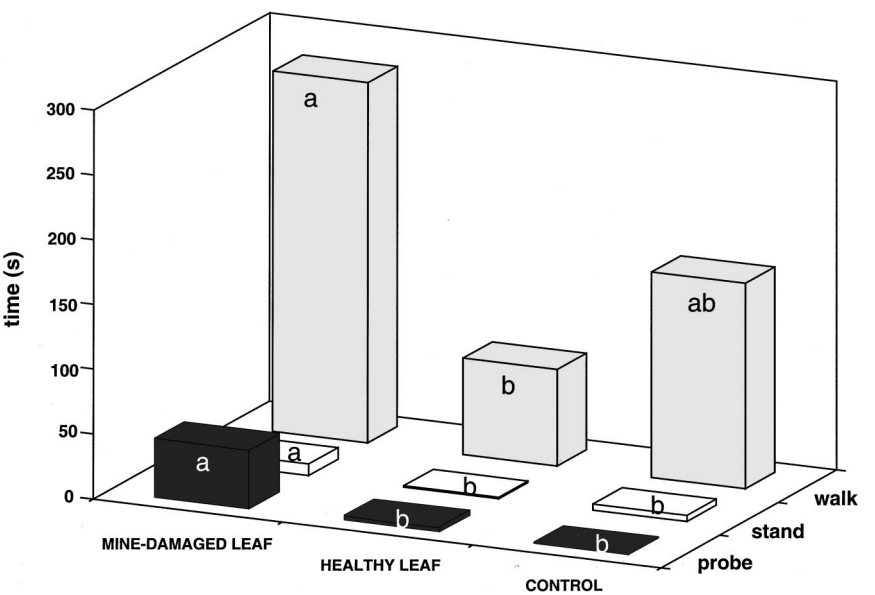

FIG. 1. Walking, standing, and probing activity of the parasitoid *P. bicolor* on mine-damaged and healthy apple leaf epidermis. Statistical differences in time duration spent for each activity on the different treatments are shown with different letters (Kruskal-Wallis ANOVA, $P < 0.05$).

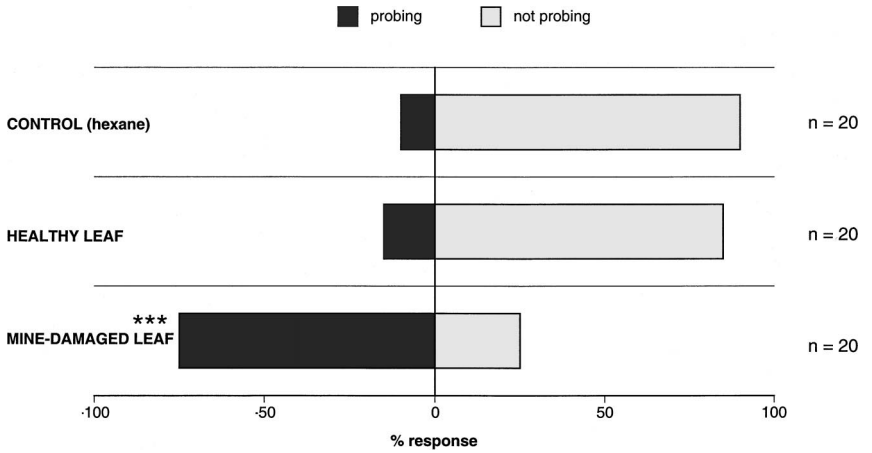

FIG. 2. Ovipositional probing response of *P. bicolor* females to hexane extracts of mine-damaged and healthy leaf epidermis. Mined-damaged vs. control: $^{***}\chi^2 = 14.7$, $df = 1$, $P < 0.001$.

In addition, no significant differences for probing and standing were observed between the healthy leaf and an empty arena (control). Only walking was similar on the mine-damaged leaf and on the control.

Experiment 2. Response to Healthy and Mine-Damaged Leaf Extracts. The ovipositional probing behavior of *P. bicolor* was triggered only by the hexane extract from mine-damaged leaves ($\chi^2 = 14.7$; $df = 1$; $P < 0.001$) (Figure 2). In contrast, the response of females to the extract from healthy leaves was similar to that of the control solvent, and substantially lower than the response to the mine-damaged leaves ($\chi^2 = 12.2$; $df = 1$; $P < 0.001$).

Experiment 3. Response to a Mixture of n-Alkanes and/or Squalene. The percent of females responding in the contact bioassay to the mixtures of synthetic compounds identified from the mine-damaged leaves is shown in Table 2. The mixture of *n*-alkanes and squalene elicited ovipositional probing behavior of *P. bicolor* at concentrations corresponding to quantities found in 1.2 cm^2 = 1 × ($\chi^2 = 20.1$, $P < 0.001$), 12 cm^2 = 10 × ($\chi^2 = 11.8$, $P < 0.001$), and 120 cm^2 = 100 × ($\chi^2 = 15.1$, $P < 0.001$) of mine-damaged leaf. The mixture of *n*-alkanes (1×) devoid of squalene did not trigger a response ($\chi^2 = 2.5$, $P > 0.05$). In contrast, squalene alone triggered a response over a range of concentrations from 30 ng to 3.1 μg. These quantities corresponded to those found in 1.2, 12, and 120 cm^2 surface area of mine-damaged leaves. The strongest response was obtained when females were offered 30 ng of squalene. This amount of squalene elicited a response that was significantly higher than to the solvent control, within the first 20 min of the bioassay.

TABLE 2. OVIPOSITIONAL PROBING RESPONSE OF FEMALE *Pholetesor bicolor* TO SYNTHETIC COMPOUNDS IDENTIFIED FROM *Phyllonorycter pomonella* HERBIVORE DAMAGED APPLE (*Malus sylvestris*) LEAVES[a]

Treatment	*n*	% females probing					
		20 min	χ^2	*P*	90 min	χ^2	*P*
Synthetic mixture (all compounds)							
0.1×	30	10	0.8	n.s.	23	2.9	n.s.
1×	30	30	5.1	≤0.05	70	20.1	≤0.001
10×	30	20	2.6	n.s.	50	11.8	≤0.001
100×	30	13	0.2	n.s.	57	15.1	≤0.001
1000×	30	7	0	n.s.	27	2.9	n.s.
synthetic mixture (1×) (excluding squalene)	30	3	0	n.s.	30	2.5	n.s.
Squalene (ng)							
3	30	7	0	n.s.	27	2.9	n.s.
15	30	7	0	n.s.	33	3.5	n.s.
30	30	30	4.0	≤0.05	63	16.1	≤0.001
310	30	13	0.2	n.s.	57	15.1	≤0.001
3100	30	20	2.3	n.s.	57	12.7	≤0.001
31000	30	7	0	n.s.	27	2.9	n.s.

[a][Concentration 1× corresponds to that given in Table 1 offered on a 1.3-cm^2 filter paper. The total observation time for each female was 90 min. χ^2 performed between treatment and control (hexane).]

Experiment 4. Squalene and n-Alkanes as Olfactory Stimuli. In the Y-tube olfactometer bioassay, no response of females towards the mixture of squalene and *n*-alkanes was observed (Figure 3), regardless of the concentration offered. This indicates that squalene is not used by *P. bicolor* as a chemical cue in habitat location.

Experiment 5. Squalene and n-Alkanes as Priming Stimuli. In the Y-tube, only females that had been given a previous contact experience on leafminer damaged plants preferred the volatiles emitted by the mine-damaged leaf versus the control ($\chi^2 = 15$; $P < 0.001$) (Figure 4). Neither a previous contact experience with the mine-damaged leaf extract nor a contact with the synthetic mixture, induced a preference towards the odor of the mined leaf.

DISCUSSION

Apple leaves infested by leafminers produce the triterpene squalene ($C_{30}H_{50}$) in quantities higher than noninfested leaves. Squalene elicits a characteristic ovipositional probing behavior of naïve *P. bicolor* which demonstrates that this chemical

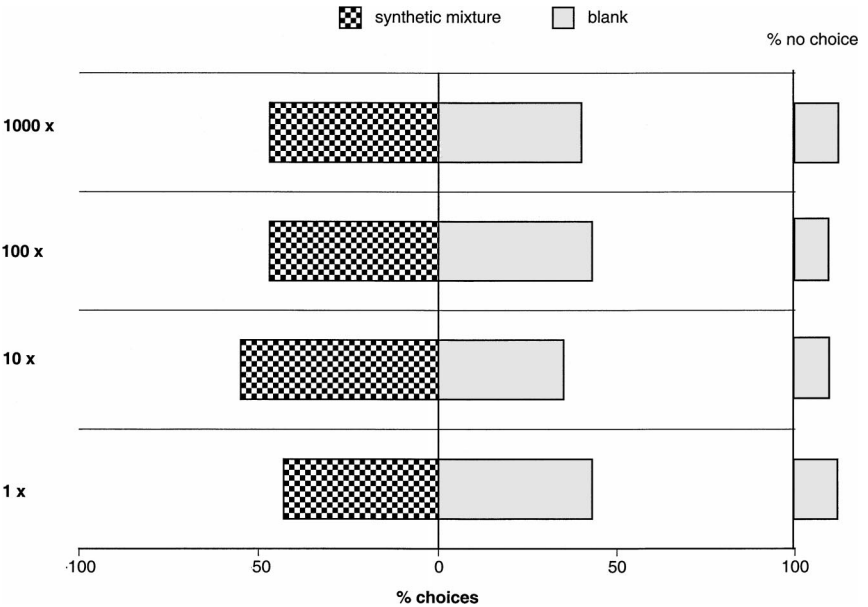

FIG. 3. Response of *P. bicolor* to the synthetic mixture of squalene and *n*-alkanes in the Y-tube olfactometer. Concentrations of synthetic mixture are indicated on the left side (dilution factor of 10). 1× corresponds to 1.2 cm^2 leaf area surface of mine-damaged leaf (see Table 1 for quantities). Percent females making no choice is indicated on the right side ($N = 30$ for each test).

is innately recognized and used by the parasitoid for host location after landing on a host-infested patch. Given that squalene cannot be synthesized by arthropods (Downer, 1985), we conclude that this compound is of plant origin. Its increased production seems to be induced by *Phyllonorycter pomonella's* herbivory. Previous reports have documented herbivore induction of terpenes in plants, and their use by parasitoids and predators for habitat location (for reviews see Rutledge, 1996 and Dicke, 1994). However, this specific triterpene has not been reported as a plant-derived semiochemical for parasitoids. Squalene in this system can be defined as a contact plant synomone for the parasitoid (sensu Dicke and Sabelis, 1988).

To our knowledge, this is the first record of squalene being extracted from apple leaves. The most closely related plant species from which squalene has been extracted is *Prunus persica* (L.) Batsch (Lin and Lin, 1992). Squalene is widely found in both plant and animal tissue, being a basic intermediate metabolite for the biosynthesis of sterols and triterpenes (Bonner, 1965). From the genesis of squalene through the mevalonate → geranyl pyrophosphate → farnesyl pyrophosphate

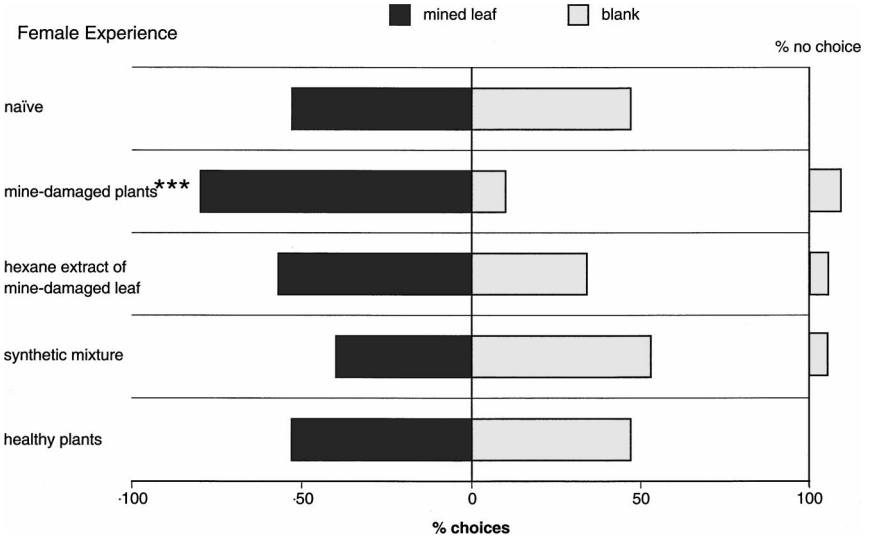

FIG. 4. Response of *P. bicolor* females to mine-damaged leaf in Y-tube olfactometer. Type of experience is indicated on left side. Percent females making no choice is indicated on the right side. Asterisks indicate a significant difference within a choice test: χ^2, $^{***}P < 0.001$ ($N = 30$ for each test).

pathway, and the subsequent steps leading to sterol production (squalene epoxidase → squalene 2,3-oxide), it is evident that squalene is an essential compound regulating plant growth (Yates et al., 1991; Simmen and Gisi, 1995). The increase of squalene in organisms such as plants, fungi, and mammals has been correlated to the exogenous inhibition of squalene epoxidase (Petranyi et al., 1984; Ryder and Dupont, 1985; Yates et al., 1991; Simmen and Gisi, 1995). The inhibition of squalene epoxidase in these organisms is shown to constrain the biosynthesis of sterols and consequently lead to growth inhibition. In our apple plant system, we observed both an increase of squalene (Table 1), and a decrease of plant growth in mine-damaged seedlings as compared to healthy seedlings (A. Dutton, personal observation). We can, therefore, hypothesize a possible direct effect of squalene on the first trophic level.

Given that squalene is an intermediate in the biosynthesis of other triterpenoids and sesquiterpenoids, it might indirectly affect the second trophic level. In fact, different plant species produce sesquiterpenoids and triterpenoids as defense compounds against pathogens (phytoalexins) and/or herbivores (Threlfall and Whitehead, 1988; Gershenzon and Croteau, 1991; Zook and Kuc, 1991). Although squalene itself has not been reported as a phytoalexin or as a toxin to herbivores, its involvement in phytoalexin biosynthesis in plant cell cultures after

fungal infection is known (Van Der Heijden et al., 1989). It would be of interest to determine if squalene has a behavioral effect on the leafminer or on other herbivore species.

Our results indicate a behavioral effect of squalene on the third trophic level. The almost threefold increase of squalene in mine-damaged leaves, with a mean value of 32 ng/1.2 cm^2 (Table 1) triggered the observed ovipositional probing behavior of the parasitic wasp *P. bicolor* (Table 2). A slightly higher amount of squalene was extracted from the mined leaf area (mean = 37 ng/1.2cm^2) (Dutton et al., 2000) as that extracted from the whole leaf (mean = 32 ng/1.2cm^2, Table 1). This indicates a possible localized increase in concentration of this substance at the site of herbivory. This finding coincides with our behavioral observation that parasitoids probe on the mined leaf area and not on healthy tissue. A similar localized response of plant compounds upon herbivory and a related parasitoid response were recently shown in the crucifer-*Pieris*-*Cotesia* tritrophic system (Horikoshi et al., 1997).

Several terpenes are known to be involved in habitat and host location of parasitoids (Rutledge, 1996). To our knowledge, this is the first report of squalene being involved in host location of a parasitoid. Behavioral effects of squalene in interactions among other organisms are known. For example, ticks of the genus *Dermacentor* ingest squalene from mammal blood and use the substance as an allomone to protect themselves against ant attack (Yoder et al., 1993). Furthermore, pollinating bats use squalene as a flower attractant (Ecroyd et al., 1995).

Enhanced responsiveness of parasitoids after a brief exposure to innately recognized cues has been demonstrated previously (Turlings et al., 1989; Lewis and Martin, 1990; Hare et al., 1997). In our system, exposing *P. bicolor* females to innately recognized cues such as squalene did not result in a subsequent attraction to leafminer infested leaves (Figure 5). However, we cannot rule out the possibility that this substance is used in associative learning (Vet et al., 1995). If such were the case, the parasitoid would need a previous exposure to the contact cue in association with plant volatiles. This can be supported by the fact that parasitoids exposed to contact and volatile cues (i.e., females given a contact experience on leafminer infested plants) subsequently were attracted to herbivore–plant volatiles.

In the Y-tube olfactometer experiments, we could not demonstrate a habitat location response to the mixture of squalene and *n*-alkanes. However, in a previous study (Lengwiler et al., 1994) and in the present experiments (Figure 4), we observed a significant attraction of parasitoids to herbivore induced leaf volatiles. It seems evident, therefore, that the volatile and contact cues used by *P. bicolor* for habitat and host location are different. In both cases the cues are of plant origin.

Acknowledgments—This project was supported by a TH grant (ETH Zurich). We thank A. Hern, C. Fornallaz, and A. Dutly for technical support with GC-MS analysis; R. Bosshard and P. Egli for

assistance with behavioral bioassays; M. Krebs and T. Christoffel for helping in rearing of plants and insects; and W. Völkl and A. Hern for their comments on an earlier draft of the manuscript.

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