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IDENTIFICATION OF A NEW BLEND OF APPLE VOLATILES ATTRACTIVE TO THE APPLE MAGGOT, *Rhagoletis pomonella*

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Abstract—Solid-phase microextraction (SPME) and gas chromatography coupled with electroantennographic detection (GC-EAD) were used to identify a new blend of volatiles from apples as the key attractants for the apple maggot fly, *Rhagoletis pomonella* (Walsh). The new five-component blend contains butyl butanoate (10%), propyl hexanoate (4%), butyl hexanoate (37%), hexyl butanoate (44%), and pentyl hexanoate (5%) compared with a previously reported seven-component mix of hexyl acetate (35%), (*E*)-2-hexen-1-yl acetate (2%), butyl 2-methylbutanoate (8%), propyl hexanoate (12%), hexyl propanoate (5%), butyl hexanoate (28%), and hexyl butanoate (10%). Volatiles from five different varieties of apple elicited reproducible and high EAD responses from *R. pomonella* antennae to the same five chemicals. In flight-tunnel choice tests involving red sticky spheres with odor sources, the new five-component blend of apple volatiles showed significantly more activity than the previous seven-component blend or the single compound, butyl hexanoate. In a field trial captures with the new five-component blend were better than with butyl hexanoate, which is currently used with commercial apple maggot monitoring spheres.

Key Words—*Rhagoletis pomonella*, apple maggot fruit fly, solid-phase microextraction, gas chromatographic–electroantennographic detection, flight tunnel, host volatiles, field experiments.

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INTRODUCTION

In fruit flies belonging to the *Rhagoletis pomonella* species complex, it has been suggested that host odors are critical for host location and fidelity and that shifts in host odor specificity may be involved in sympatric speciation within the group (Feder and Bush, 1989; Frey and Bush, 1990; Bush, 1993, 1994; Feder et al., 1994). One of the most studied species in the complex is the apple maggot, *R. pomonella* (Walsh), which occurs in North America on native hawthorne (*Crataegus*), the natural host, and on introduced apple (Chapman and Lienk, 1971; Fletcher and Prokopy, 1991). Prokopy et al. (1973) showed that in a field situation *R. pomonella* flies were attracted to the odor of apples, which led to the characterization of a seven-component mix (Fein et al., 1982) containing hexyl acetate, (*E*)-2-hexen-1-yl acetate, butyl 2-methylbutanoate, propyl hexanoate, hexyl propanoate, butyl hexanoate, and hexyl butanoate from apple extracts (see Carle et al., 1987). This blend elicited positive behavioral responses in olfactometer tubes and a wind tunnel (Fein et al., 1982; Averill et al., 1988). Field tests showed also that the chemical mix was an effective attractant (Reissig et al., 1982).

Here, we present evidence that a new five-component blend of volatiles from apples proved to be more attractive than the previous blend to *R. pomonella* flies in the laboratory and in the field. The new blend was identified by using a combination of two techniques in the analysis of headspace volatiles from fruit: solid-phase microextraction (SPME; Supelco) (see Bartelt, 1997) combined with gas chromatography and electroantennographic detection (GC-EAD).

METHODS AND MATERIALS

Insects. Adult *R. pomonella* were obtained from a laboratory colony maintained on Red Delicious apples (Neilson and McAllen, 1965). Pupae were kept in an environmental chamber with 24°C day and 22°C night temperatures, 16L : 8D photoperiod, and 35% relative humidity. Within one week after fly emergence, the adults were transferred to a second chamber with 23°C constant temperature, 50% relative humidity, and 16L : 8D photoperiod. Adults were maintained on an artificial diet (water, sugar, vitamins, casein hydrolysate, and salt mixture), and larvae were reared on Red Delicious apples. Adult flies at 0–7 days old and 2–3 weeks old were used for GC-EAD analyses and flight-tunnel behavior tests, respectively.

Fruits. Five different apple varieties (Empire, Crispin, Cortland, Macintosh, and Red Delicious) were collected from uninfested orchards near Geneva, New York, in the 1996 harvest season and stored at 4°C. The fruits were taken from cold storage and placed in the sampling containers (see below) at room

temperature at least 1 hr before the SMPE sampling began. At least four samples were collected from each variety.

SPME Sampling. Single apples were placed in 500-ml clear straight-sided jars with Teflon liner screwcaps (Wheaton, Millville, New Jersey). A polydimethyl siloxane-coated SPME fiber (film thickness 100 μm; Supelco Inc., Bellefonte, Pennsylvania) was conditioned in the GC injector (250°C) for 5 min and then passed through the small hole on the cap into the jar. The fiber was exposed for 10 min to absorb the volatiles, and the needle was then pierced into the GC injection port for 2 min to desorb the analytes.

Electrophysiological Recordings. Coupled GC-EAD analyses were performed as described before (Zhang et al., 1997). A Hewlett Packard 5890 Series II gas chromatograph equipped with a nonpolar SE-30 Econo-Cap capillary column (30 m × 0.25 mm ID, 0.25 mm film thickness; Alltech Associates, Inc., Deerfield, Illinois) or a polar Stabilwax capillary column (30 m × 0.25 mm ID, 0.25 mm film thickness; Restek Corp., Bellefonte, Pennsylvania) in the splitless mode was used for GC-EAD analysis. The oven temperature was programmed at 50°C for 2 min, then 10°C/min until 250°C, and held for 20 min. Injector and detector temperature were set at 250°C. Nitrogen was the carrier gas with a flow rate of 2 ml/min. Septum purge flow rate was set at 3 ml/min and the split vent flow rate at 2 ml/min. All of the gas source pressures were 276 kPa (40 psi), and initial column head pressures were 138 kPa (20 psi). The column effluent was split 1 : 1 in the oven of the flame ionization detector and the EAD. A whole head was removed from a fly and both antennae were positioned between two gold wire electrodes, which were immersed in saline-filled wells in a small acrylic holding station. The fly's head was placed in one well and the tips of both antennae maneuvered to make contact with the saline in the other well. The temperature of the antennae on the acrylic station was maintained at about 5°C by flushing 0°C water from a benchtop refrigerated circulator (RTE-100, NESLAB Instruments, Inc., Portsmouth, New Hampshire) through the insulation layer of the modified condenser containing the acrylic station mounted on top of the GC. An HP 3390 A integrator was used for the EAD recordings. A minimum of 10 different antennal pairs (2–3 runs/pair) were used to analyze volatiles from each cultivar.

GC-mass spectrometry (GC-MS) was carried out with a Hewlett Packard 5890 gas chromatograph coupled to an HP 5970B Mass Selective Detector (EI) with an SE-30 capillary column, but with helium as the carrier gas. The oven temperature was programmed at 40°C for 5 min, then 5°C/min to 250°C, and held for 20 min.

Chemicals. All synthetic compounds were purchased from either Pfaltz & Bauer, Inc., Stamford, Connecticut, or Penta International Corp., West Caldwell, New Jersey, and purities were >98% based on results with the capillary GC (FID).

TABLE 1. APPLE MAGGOT ATTRACTANT BLENDS USED IN FLIGHT TUNNEL (FT) AND FIELD TESTS

Old blend		New		
Compound	FT (%)	Compound	FT (%)	Field
Hexyl acetate	35%			
(*E*)-2-Hexen-1-yl acetate	2%			
		Butyl butanoate	10%	2
Butyl 2-methylbutanoate	8%			
Propyl hexanoate	12%	Propyl hexanoate	4%	1
Hexyl propanoate	5%			
Butyl hexanoate	28%	Butyl hexanoate	37%	10
Hexyl butanoate	10%	Hexyl butanoate	44%	10
		Pentyl hexanoate	5%	1

Flight Tunnel. Responses of AM flies to host volatiles were measured in a sustained-flight tunnel (Glover et al., 1987). Outside dimensions of the tunnel were 183 cm long, 61 × 61 cm square; the tunnel was constructed of glass and had a metal frame. Diffuse overhead light was provided by four Vita-Lite fluorescent bulbs (Fein et al., 1982), as well as fluorescent lighting from the ceiling of the room housing the tunnel. The tunnel was housed in a room with temperature and relative humidity controls and an exhaust system from the tunnel to the outside of the building. Flight-tunnel conditions were: 25–27°C, 50–70% relative humidity, 40 cm/sec wind speed, and 1200 lux light intensity.

The experimental design involved a choice test, modified after Fein et al. (1982) and Averill et al. (1987), incorporating red spheres (7.5 cm; Gempler's Inc., Mt. Horeb, Wisconsin) coated with Tangle trap. One sphere served as a blank or as a second odor source. The two spheres were hung from the roof of the upwind end of the tunnel by metal wires so that they were 25 cm above the tunnel floor and 35 cm apart. Treatments included the seven-component blend, the new five-component blend, and the single compound butyl hexanoate. The single compound or the mixtures (see Table 1) were prepared in hexane at concentrations so that 100 μl of solution were applied for each load rate to red rubber septa (Thomas Scientific, Swedesboro, New Jersey, Cat. No. 1780J07) that had been acetone-washed. Sources were prepared 30 min prior to a test and were clipped onto the metal rods holding the spheres, 1 cm above a sphere. Blank spheres contained rubber septa treated with hexane.

Two flight tunnel experiments were conducted. The first involved choice tests between a blank sphere and one with 10, 30, 100, or 300 μg of the seven-component blend, the new five-component blend, or the single compound butyl hexanoate. The second involved choice tests between spheres containing 100-μg doses of the five-component blend and the seven-component blend or butyl hex-

anoate alone. For each test, 50 flies (mixed sex, 2–3 weeks old) were placed in glass vials (10–15 flies/vial). The flies were selected from holding cages located in a separate, environmentally controlled, room, taken to the flight tunnel, and allowed to acclimate for 5 min. The odor source(s) were then placed in the tunnel and the flies released. Fresh volatile sources and red spheres were used for each test. Tests ran for 1 hr, at which time the number of captured flies was recorded. For all tests, six replicates were performed, with the position of the spheres alternated over the six replicates.

Field Experiments. A field test involving the new apple blend and butyl hexanoate, the commercial treatment, was conducted during July, August, and September, 1997, in Massachusetts. The test involved eight commercial apple orchards, divided into two groups of four orchards each, group 1 trees were baited with butyl hexanoate for the first half of the test (four weeks), and then with the new apple blend (four weeks), whereas group 2 trees were baited with butyl hexanoate throughout the season (eight weeks). The new apple blend, a 2:2:10:10:1 mixture of butyl butanoate, propyl hexanoate, hexyl butanoate, butyl hexanoate, and pentyl hexanoate, was loaded to capacity in 2-dram polyethylene vials (Reissig et al., 1982), similar to the butyl hexanoate lures. The proportions approximated the average values obtained from integration of the total ion mass spectra obtained with a temperature program that gave separation of all compounds from the five apple varieties (see Table 2 below). For each orchard, there were three blocks of 49 trees in a 7×7 arrangement. Each 49-tree block was at the corner of a larger block, the remainder of which was sprayed three times with insecticide for apple maggot fly control during the summer of 1997. Thus, for each 49-tree block, two sides faced adjacent woods and two sides faced sprayed trees. Each of the 26 perimeter trees in each 49-tree block received a baited sticky red sphere. All spheres were in place in early July. Counts were made twice during each half of the test.

Statistical Analysis. Mean capture rates for the six replicates in the flight tunnel and for the two treatment groups in the field were compared by using a two-sample independent groups t test, with GB-STAT for Macintosh ($P < 0.05$). The analysis included tests for normality and homogeneity of variance, according to the GB-STAT program.

RESULTS

Identification of Key Host Volatiles from Apples. For each apple variety, SPME headspace sampling and GC-EAD analysis was conducted at least four times by using male and female antennae. Although the GC profiles varied among the apple varieties, SPME sampling of five different varieties consistently revealed the same four significant antennal responses to the volatiles on

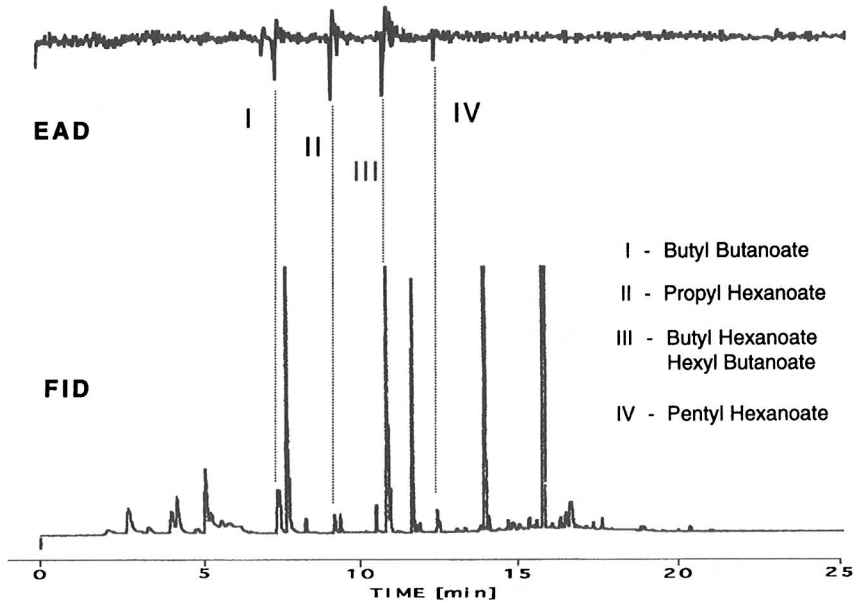

FIG. 1. Simultaneous responses of FID and EAD using the antennae of female *R. pomonella* to SPME desorbed apple volatiles on an SE-30 capillary column. The trace is from an Empire apple, but is representative of the five varieties sampled.

the SE-30 and the Stabilwax capillary columns (Figure 1). No significant difference in antennal sensitivity was observed between male and female antennae. The corresponding EAD active compounds were identified as butyl butanoate (peak 1), propyl hexanoate (peak 2), butyl hexanoate and hexyl butanoate (peak 3), and pentyl hexanoate (peak 4), respectively, by comparison of mass spectra and GC-MS retention times on both polar and nonpolar capillary columns with synthetic standards. Although butyl hexanoate and hexyl butanoate cochromatographed as the third EAD active peak under the GC conditions used, the two compounds could be separated by using a lower temperature program without any loss in EAD response. These two compounds made up about 80% of the total five active esters and usually elicited the strongest GC-EAD response from headspace volatiles. The relative ratios of the five esters in the different apple varieties, as determined by SPME and GC-MS, are listed in Table 2. Activity of the five esters was confirmed by strong antennal responses elicited by 1 ng or less of synthetic compounds.

Flight Tunnel Experiment 1. Upon exposure to the odor stream, flies emerged quickly from the vials and dispersed in the downwind end of the tunnel. Capture on the red sphere occurred via a series of short flights biased in

TABLE 2. RATIO OF ACTIVE CHEMICALS FROM DIFFERENT APPLE VARIETIES AS DETERMINED BY INTEGRATION OF GC-MS TOTAL ION CURRENT[a]

Chemicals	Apples					Mean ± SD
	Em	Cr	Co	Mc	RD	
Butyle butanoate	4.3	9.4	8.4	24	15.9	12.4 ± 7.7
Propyl hexanoate	6.7	3.5	3.5	5.8	6.3	5.2 ± 1.5
Butyl hexanoate	83	75	17	25	25	45 ± 31.3
Hexyl butanoate	17	25	83	75	75	55 ± 31.1
Pentyl hexanoate	9.8	4.8	8.4	3.5	4.5	6.2 ± 2.7

[a]Apples: Em = Empire, Cr = Crispin, Co = Cortland, Mc = Macintosh, RD = Red Delicious. Ratios calculated on the basis of butyl hexanoate and hexyl butanoate, which cochromatograph on the GC-EAD, summing to a value of 100. At least four samples were collected from each variety.

the upwind direction, with less than 5% of the flies exhibiting directed flights at distances of 1.0 m or greater to the sphere. Short flights were interspersed with bouts of walking and grooming. Short flights, grooming, walking, and encounters with other flies appear to be basic components of normal arousal behavior of the flies during the photophase. The presence of odor increased the rate of net upwind movement bringing adults to the upwind end of the tunnel, where in numerous instances short directed flights were made to the spheres. The success of the assay may have been due to the small height and width of the tunnel (61 × 61 cm), which kept the flies within or close to the odor space for the duration of the test.

In the first experiment, adult responses were assessed to one of four doses (10, 30, 100, or 300 μg) of the new or old apple blend, or butyl hexanoate, against a blank sphere. The results of these choice tests (Figure 2), show that mean captures on the blank spheres were consistent over the entire set of tests, averaging 6 ± 2 flies/sphere, and with no significant differences among the blanks for any dose/treatment combination. With the 10-μg sources, there was no significant difference in mean captures among the three treatments and the blanks. Greatest response at 30, 100, and 300 μg was observed with the new apple volatile blend. Mean values with the new blend were significantly greater than controls at 30 μg, and at 100 and 300 μg this was true for all three treatments compared with the blanks.

Flight Tunnel Experiment 2. Adult responses were assessed using the 100-μg dose in choice tests involving either the new five-component vs. the seven-component apple blend or the new blend vs. butyl hexanoate. The 100-μg dose was selected based on differences in mean captures observed in experiment 1. The results (Figure 3) show that in each choice test significantly more flies were captured with the new apple blend.

Field Trial. The results of field tests comparing fly responses to the new

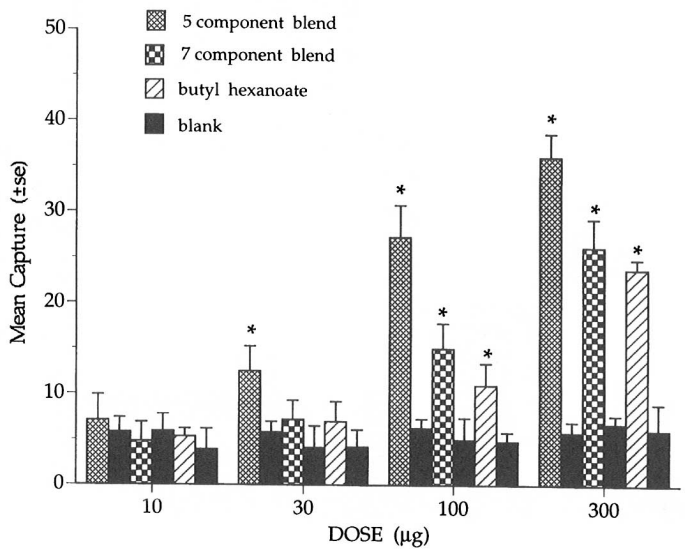

FIG. 2. Responses of adult *R. pomonella* in a sustained-flight tunnel in choice tests involving four doses (10, 30, 100, and 300 μg on rubber septa) of the new five-component blend, the seven-component blend, or butyl hexanoate on a sticky red sphere versus a blank red sphere. Values are mean capture ± SE, $N = 6$ replicates of 50 flies for each choice test. Bars within each choice test with different letters indicate significant differences, by using a t test ($P < 0.05$).

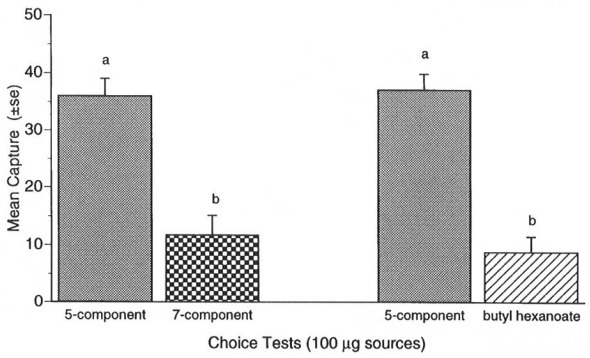

FIG. 3. Responses of adult *R. pomonella* in flight tunnel choice tests to the new five-component blend versus the seven-component blend (left), and the new blend versus butyl hexanoate (right). Values are mean ± SE, $N = 6$ replicates of 50 flies for each test. Bars within each choice test with different letters indicate significant differences, by using a t test ($P < 0.05$).

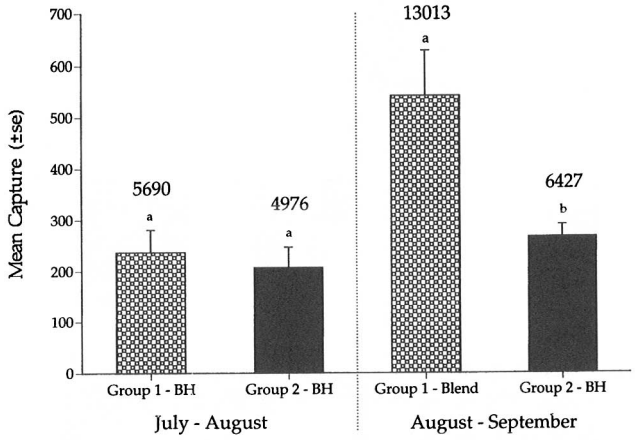

FIG. 4. Mean capture (± SE) of adult *R. pomonella* in field trials comparing sticky red spheres baited with two butyl hexanoate treatments (groups 1 and 2 in July–August on the left), and the new five-component blend (group 1) versus butyl hexanoate (group 2) in August–September (on the right). Numbers above each bar are the total number of flies captured. Bars in each test with different letters indicate significant differences, by using a t test ($P < 0.05$).

apple blend and butyl hexanoate are shown in Figure 4. During July and early August, tests were performed in which red spheres in both groups of orchards were baited with butyl hexanoate. There was no significant difference in mean captures between the two groups. In late August–September, red spheres in orchards in group 1 were baited with the new apple blend, and mean capture in this group was significantly greater than in the orchards in group 2 baited with butyl hexanoate.

DISCUSSION

We have identified a new five-component blend of volatiles from apples that contains three compounds (propyl hexanoate, butyl hexanoate, and hexyl butanoate) in common with the previous seven-component blend, as well as two new compounds, butyl butanoate and pentyl butanoate. The esters in the new blend were identified from five apple varieties and gave consistently high levels of EAD activity. Flight-tunnel experiments showed that the new blend was more attractive to *R. pomonella* adults than the previously identified blend of esters or the single compound, butyl hexanoate; field experiments showed that the new mix was superior to butyl hexanoate.

Successful characterization of apple volatiles resulted from the use of SPME

and GC-EAD. The SPME technique deletes the solvent extraction step, allowing for collection of volatiles directly from the headspace of the fruit in quantities that are consistent with their release rate in the field and without interference from contaminating compounds typically found in collections that use more traditional methods (see Bartelt, 1997; Agelopoulos and Pickett, 1998; Matich et al., 1996, for recent critical discussions of the SPME technique). Often, high quantities of fruit volatiles are collected by other methods, with the GC-EAD analysis exhibiting mediocre responses to many compounds and no clear evidence for the key components. With our experimental conditions, the amount of each electrophysiologically active compound collected by SPME was in the range of 1–20 ng based on GC analysis.

One potential criticism of our analysis concerns the use of apples that were picked and kept in cold storage, or, in general, the problem of changing volatile profiles as a function of age or picking. In a previous study, Carle et al. (1987) analyzed volatile profiles from a number of apple varieties and showed that, whereas many of the same esters occurred in each variety, there was significant variability in the profiles for each variety and that these profiles changed quantitatively over the growing season and with respect to storage conditions. Sampling the volatiles, however, with the SPME technique and analysis of these volatiles with GC-EAD showed that the same key compounds are found, regardless of the qualitative and quantitative differences in the volatile profiles.

The sustained-flight tunnel was used to assay the identified compounds for behavioral activity, with a 1-hr period proving sufficient to test the ability of *R. pomonella* flies to respond to and discriminate between host odors. The sustained-flight tunnel had been used previously with *R. pomonella* by Fein et al. (1982) for the characterization of the seven-component apple blend and by Aluja et al. (1993) to study the effects of air speed and volatiles on *R. pomonella* behavior. Although our flight tunnel did not incorporate structures simulating foliage (as in Aluja et al., 1993), the flies exhibited patterns of behavior similar to those that have been observed in field cage experiments demonstrating the effect of host volatiles on inter- and intratree flight behaviors (Aluja and Prokopy, 1992, 1993; Aluja et al., 1993; Green et al., 1994).

The new five-component blend of esters also was attractive in apple orchards, capturing more *R. pomonella* on red sticky spheres than equivalent amounts of butyl hexanoate alone. The latter compound is often used in conjunction with red spheres as a monitoring tool in commercial orchards.

A major goal of our research is to address questions about the role of host odors in maintaining host specificity in *Rhagoletis* flies and in the proposed processes involving host shifts via sympatric speciation. The dependence of the mate recognition system on the linkage between host choice and mate finding makes it possible for variation in traits affecting host utilization to act as genetically based, premating isolation mechanisms (McPheron et al., 1988; Feder et al.,

1989, 1990; Bush, 1993, 1994; Berlocher et al., 1993; Smith and Bush, 1997). With the development of the SPME and GC-EAD techniques, it should be possible to identify the key host volatiles for species within the *R. pomonella* group and to test in greater detail questions concerning the role of host odors.

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