Journal of Chemical Ecology, Vol. 29, No. 10, October 2003 (© 2003)

IDENTIFICATION OF HOST FRUIT VOLATILES FROM FLOWERING DOGWOOD (*Cornus florida*) ATTRACTIVE TO DOGWOOD-ORIGIN *Rhagoletis pomonella* FLIES

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(Received April 16, 2003; accepted June 18, 2003)

Abstract—Solid-phase microextraction and gas chromatography coupled with electroantennographic detection were used to identify volatiles from fruit of flowering dogwood, *Cornus florida*, as key attractants for *Rhagoletis pomonella* flies originating from dogwood fruit. A six-component blend containing ethyl acetate (54.9%), 3-methylbutan-1-ol (27.5%), isoamyl acetate (0.9%), dimethyl trisulfide (1.9%), 1-octen-3-ol (9.1%), and β-caryophyllene (5.8%) was identified from flowering dogwood fruit that gave consistent EAD activity. In a flight tunnel assay there was no significant difference in the response of individual dogwood flies exhibiting upwind anemotactic flight to volatile extracts from dogwood fruit and the six-component synthetic mixture. Dogwood flies also displayed significantly greater levels of upwind flight to sources with the dogwood volatile blend than with previously identified volatile blends from domestic apple or hawthorn fruit. Selected subtraction assays showed that the three-component mixture of 3-methylbutan-1-ol, 1-octen-3-ol, and β-caryophyllene elicited levels of upwind flight to the source equivalent to the six-component mixture. Our study adds to previous ones showing that populations of *Rhagoletis pomonella* flies infesting apple, hawthorn, and flowering dogwood fruit are attracted to unique mixtures of fruit volatiles, supporting the hypothesis that host fruit odors could be key traits in sympatric host shifts and establishing host fidelity within members of the *Rhagoletis pomonella* species complex.

Key Words—Solid-phase microextraction gas chromatographic electroantennographic detection, flight tunnel, *Rhagoletis*, flowering dogwood, *Cornus florida*, fruit volatiles.

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INTRODUCTION

The *Rhagoletis pomonella* species group (Bush, 1966; Berlocher, 2000) is a complex of closely related races, sibling species, and one morphologically distinct species (*R. cornivora*) that infest different fruits of host plants in North America. The apple maggot fly, *R. pomonella*, exists as two host races, one on native hawthorn (*Crataegus*) and one on introduced apple (McPheron et al., 1988; Feder, 1998). A closely related fly to the *R. pomonella* host races occurs on flowering dogwood, *Cornus florida* (Berlocher et al., 1993; Berlocher, 1999, 2000). Genetic analysis and life history data suggest that the fly infesting flowering dogwood may be of species status, even though some interpopulation gene flow is occurring (Berlocher, 1999, 2000).

A key feature of the biology of *Rhagoletis* flies is that mating occurs on or near host fruit that females will oviposit in, a concept called "host fidelity" (Feder, 1998). We have been investigating the role of host fruit odors in establishing and maintaining host fidelity within the group (Zhang et al., 1999; Berlocher and Feder, 2002; Nojima et al., 2003). Previous studies have shown that apple-origin flies are attracted to the odor of apples (Prokopy et al., 1973), and to synthetic compounds isolated from apple fruit (Fein et al., 1982; Reissig et al., 1982; Averill et al., 1988; Zhang et al., 1999). More recently we showed that hawthorn-origin flies respond to a blend of hawthorn fruit volatiles that is different from the mixture characterized from domestic apple fruit (Nojima et al., 2003). Here, we present evidence that flowering dogwood flies respond behaviorally to yet a different blend of volatiles, identified from dogwood fruit, using solid-phase microextraction (SPME) (Supelco™) combined with gas chromatography and electroantennographic detection (GC-EAD) for the analysis of headspace volatiles from fruit.

METHODS AND MATERIALS

Insects. Dogwood-origin larvae were collected from fruit at Granger, Indiana. Post-diapause pupae (Feder et al., 1994) were shipped to the lab in Geneva, New York, and kept in an environmental chamber at 23–24°C, 16L:8D photoperiod, and 65–70% relative humidity. Adults were maintained in similar conditions on an artificial diet (Fein et al., 1982; water, sugar, vitamins, casein hydrolysate, and salt mixture). Adult flies at 0–7 and 10–21 days old were used for GC-EAD analyses and flight-tunnel behavior tests, respectively.

Fruit. Dogwood fruit were collected at Granger and Racoon Lake Recreational Area, Indiana, during September and October 2000 and 2001 and shipped to the Geneva lab. Fruit were held in cold storage (4°C, never more than 2 weeks) until sampled. Fruit were taken from cold storage and placed in the sampling containers (see below) at room temperature at least 1 hr before SPME sampling. A minimum of nine SPME samples were taken from each fruit collection.

Adsorbent Sampling. A glass chamber (3 l; Analytical Research Systems, Gainesville, Florida) with a charcoal filter tube in the inlet was used for collection of volatiles. Fruit were placed in the apparatus, and the volatiles from the head-space were drawn (about 0.8 l/min) by a vacuum pump onto ORBO solvent de-sorption tubes (150-mg active coconut charcoal, 20/40 mesh, 4 mm i.d., 70-mm length; Supelco Inc., Bellefonte, Pennsylvania) in the outlet. Two tubes were set in series to provide a backup in case one tube broke. Adsorbent samplings were made from 1 kg of fruit for 9 d at room temperature. The ORBO tubes were changed every 3 d. Volatiles were eluted with 1-ml methylene chloride (nanograde, Mallinckrodt), dried over anhydrous sodium sulfate, and then combined. Extracts were kept in a freezer (-20°C) and subjected to GC-MS analysis and flight tunnel bioassay.

SPME Sampling. A glass jar (500 ml) with Teflon liner screwcap (Wheaton, Milliville, New Jersey) was used for a sampling container. Fruit (about 150 g) were placed in the container at least 1 hr before the SPME sampling. A carboxen-polydimethylsiloxane coated SPME fiber (film thickness 85 μm; Supelco Inc., Bellefonte, Pennsylvania) was used for head-space volatile collection. The fiber was conditioned in the GC injector (280°C) for 20 min, and then passed through the small hole on the cap into the head-space of the jar. After 10–30-min exposure, collected volatiles on the fiber were immediately subjected to GC-EAD analysis. For the chemical analysis of the volatiles with GC-MS, SPME samplings were made for a longer time (a few hours to overnight) if necessary.

Coupled Gas Chromatographic–Electroantennographic Detection (GC-EAD) Analysis. An Hewlett Packerd 5890 series II gas chromatograph equipped with a nonpolar SPB-1 capillary column (30 m $\times$ 0.25 mm i.d., 0.25-mm film thickness; Supelco Inc., Bellefonte, Pennsylvania) or a polar EC-WAX Econo-Cap capillary column (30 m $\times$ 0.25 mm i.d., 0.25-mm film thickness; Alltech, Deerfield, Illinois) in the splitless mode was used for GC-EAD analysis. For SPME sample injection, a 0.75-mm i.d. glass inlet liner (Supelco Inc., Bellefonte, Pennsylvania) was used, while for liquid sample injection a 4-mm i.d. liner was used. Nitrogen was the carrier gas at a head pressure of 138 kpa (flow rate, 2.0 ml/min). The time for splitless injection was 0.25 min for SPME samples and 1.0 min for liquid samples. Oven temperature was programmed for 40°C for 2 min, and then increased at 15°C/min to 250°C, where it was held for 10 min. Injector and detector temperature were set at 280°C. Septum purge flow rate was set at 3 ml/min and total flow rate at 60 ml/min.

The column effluent was combined with nitrogen makeup gas (30 ml/min) and then split 1:1 to the flame ionization detector (FID) and EAD with a TCD capillary column adapter to facilitate ease in changing columns (Nojima et al., 2003). The EAD outlet was secured in a charcoal-filtrated and humidified air stream, which was refrigerated by a modified condenser flushed with 0°C water, flowing at about 500 ml/min over a *Rhagoletis* fly antennal preparation. A fly head, separated at the prothorax, was mounted on a custom acrylic holder, equipped with

gold wire electrodes (Nojima et al., 2003). Drosophila ringer solution (46 mmol NaCl, 182 mmol KCl, 3 mmol $CaCl_2$, and 10 mmol Tris-HCl at pH 7.2) was used for making electrical conductivity between electrodes and antennae. The holder was placed inside of a cooling condenser and maintained about 5°C.

The output signal from the antennae was amplified by a customized single step high-input impedance DC amplifier at ×100 (Nojima et al., 2003). The signal was recorded on an HP 3390 A and synchronized with a GC integrator. As we found previously with hawthorn-origin flies (Nojima et al., 2003), preliminary experiments showed no significant qualitative differences in EAD responses between apple-origin *R. pomonella* flies from our lab colony and dogwood flies from nature, or between sexes within each race. Because of limited availability of dogwood flies needed for behavioral testing, key host volatiles from dogwood fruit were first estimated using the lab colony flies reared on apple, and then the results were confirmed with dogwood flies from nature.

Chemical Analysis. GC-MS analyses were performed as in Nojima et al., (2003) on a Shimadzu GC-17A equipped with a nonpolar DB-1ms capillary column (30 m × 0.25 mm i.d., 0.25-mm film thickness; J&W Scientific, Folsom, California) or a polar EC-WAX Econo-Cap capillary column (30 m × 0.25 mm i.d., 0.25-mm film thickness; Alltech, Deerfield, Illinois) and coupled to a Shimadzu QP 5050A quadrupole mass spectrometer running in the EI (electron ionization at 70 eV) scan mode. Helium was the carrier gas at an initial head pressure of 58 kpa at constant flow rate (1.0 ml/min). Oven temperature was programmed for 40°C for 2 min, increased at 15°C/min to 250°C, and held for 10 min. The injector and interface temperature were set at 280 and 260°C, respectively. Volatiles were identified by mass spectral matches to library spectra as well as by retention time matches to available known standards. The EAD activity of compounds was verified by the GC-EAD analysis with authentic standards. Quantification of the relative ratios of the EAD active compounds was made from the absorbent collections. The compounds were quantified from their ion abundances or FID responses of GC-MS or GC analyses according to the standard curves made from each authentic standard.

Chemicals. 3-Methylbutan-1-ol, isoamyl acetate, 1-octen-3-ol (a racemic mixture), and ethyl acetate were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin), and purities were >98%. Dimethyl trisulfide was purchased from Acros Organics (Pittsburgh, Pennsylvania), and purity was >98%. β-Caryophyllene, a mixture of β and α isomers at a ratio of 91:9 was purchased from ICN Biomedicals, Inc. (Irvine, California). Net proportion of isomers was >95% based on GC analysis.

Flight Tunnel. The response of flies to host fruit volatiles was measured in a sustained-flight tunnel (Nojima et al., 2003). Flight tunnel conditions were 23–24°C, 50–70% relative humidity, 35 cm/sec wind speed, 1000-lux light intensity. Adult Flies were tested between the 3rd and 11th hours of the 16 hr photophase period (6 A.M. to 10 P.M. EST). Adult flies (mixed sex, 10–21 days old) were

selected from holding cages located in a separate, environmentally controlled room, placed singly in glass vials, taken to the room housing the flight tunnel, and allowed to acclimate for 15 min. Individual flies were transferred to a screen release cage, which was placed on a screen stand inside the flight tunnel. The stand was positioned 1 m downwind of a red sphere used as a release point for volatile fruit blends (see below). The release cage was open at one end, with the open end facing in the upwind direction. Flies were given 1 min to respond, and scored for the following behaviors: 1) taking flight = flight from the release cage (100 cm from the source, may or may not be odor-stimulated); 2) upwind flight = odor-stimulated upwind flight oriented toward the sphere for at least 20 cm (80 cm from the source); and 3) reaching sphere = odor-stimulated upwind flight to and landing on the red sphere holding the source (0 cm). Flies were recovered after testing and used again, but never on the same day or for more than four trials.

Solutions of volatile extracts in methylene chloride, or synthetic compounds prepared in hexane, were applied to red rubber septa (Thomas Scientific, Swedesboro, New Jersey, Cat. No. 1780J07) that had been acetone-washed. The rubber septum was attached to a 7.5-cm red plastic sphere (Gempler's Inc., Mt. Horeb, Wisconsin) and hung from the ceiling at the upwind end of the tunnel. The proportions of chemicals in the dogwood blend are shown in Table 1. For the hawthorn blend (Nojima et al., 2003), the chemicals and proportions were ethyl acetate (2000), 3-methylbutan-1-ol (100), isoamyl acetate (40), 4,8-dimethyl-1,3(*E*),7-nonatriene (2), butyl hexanoate (1), and dihydro-β-ionone (2). For the apple blend (Zhang et al., 1999), the chemicals and proportions were butyl butanoate (10), propyl hexanoate (4), butyl hexanoate (37), hexyl butanoate (44), and pentyl hexanoate (5). For comparative purposes, the reported dosage of the dogwood synthetic blend and volatile extract, and the hawthorn blend, reflects the amount of 3-methylbutan-1-ol, with the other components added in the proportions

TABLE 1. RATIO OF ACTIVE CHEMICALS FROM FLOWERING DOGWOOD FRUIT AS DETERMINED BY INTEGRATION OF GC-FID, GC/MS TOTAL ION CURRENT USING ADSORBENT SAMPLE[a]

Chemicals	GC/FID; GC/MS (%)	FT (%)
Ethyl acetate	54.9	200
3-Methylbutan-1-ol	27.5	100
Isoamyl acetate	0.9	3
Dimethyl trisulfide	1.9	7
1-Octen-3-ol	9.1	30
β-Caryophyllene	5.8	20

[a] FT indicates the percentage of each compound in the synthetic blend used in the flight tunnel.

shown above. The reported dose of the apple mixture reflects the total amount of the compounds. Sources were prepared 60 min prior to a test, and were clipped onto the bottom of the red sphere. Blank spheres used for control experiments contained rubber septa treated with hexane. Fresh sources and red spheres were used for each test period.

Experiment 1. The response of dogwood flies to a 50-μg dose of the natural volatile extract from dogwood fruit was compared with a 50- and 200-μg dose of the complete six-component dogwood fruit blend identified from SPME-EAD analysis. The 50-μg dose was selected on the basis of analysis of the extract showing that it contained approximately 0.25 μg/μl 3-methylbutan-1-ol, resulting in 50 μg of the compound when 200 μl of the solution was applied to the rubber septum.

Experiment 2. Dogwood flies were tested to 200-μg dose of the six-component dogwood blend, the 6-component hawthorn blend (Nojima et al., 2003), and the five-component blend identified from apple (Zhang et al., 1999).

Experiment 3. The response of dogwood flies to a 200-μg dose of the six-component dogwood blend was compared with selected treatments in which single or multiple compounds were removed from the complete blend.

In all experiments, 10–15 flies were tested each day to each treatment, until a minimum of 40 different flies had been tested/treatment (with the exception of treatments 6 and 7, experiment 3, where $N = 15$). Over the course of the studies, a total of 35 flies also were tested to a control red sphere containing a solvent treated rubber septum.

Statistical Analysis. The total number of flies in each treatment that exhibited each behavior in the flight sequence was converted to a percent value for graphical display. Selected 2×2 comparisons were made *a posteriori* between values for different treatments within each experiment, using Fisher's exact test, according to the JMP statistical analysis program ($P < 0.05$).

RESULTS

Identification of Key Host Volatiles from Dogwood Fruit. A total of seven different fruit samples from Granger and Racoon Lake, Indiana, were analyzed using SPME with GC-EAD. Three fruit samples were collected from September to October 2000, and four during the same months in 2001. A total of 229 different antennal pairs were tested for the study (1–8 runs/pair; 129 female and 68 male colony apple-origin flies; 19 female and 13 male dogwood flies). Comparison of EAD samples from dogwood fruit with the Indiana dogwood flies and New York apple-origin flies allowed us to determine the key group of volatiles that consistently gave EAD responses. The corresponding six active compounds were identified as ethyl acetate, 3-methylbutan-1-ol, isoamyl acetate, dimethyl trisulfide, 1-octen-3-ol, and β-caryophyllene.

A single adsorbent extract of volatiles from whole ripe fruit collected at Racoon Lake in September 2000 was made for quantitative analysis of key volatiles and flight tunnel analysis. The relative ratio of the EAD active compounds in this sample, estimated with GC-FID and GC-MS, are listed in Table 1. Flight tunnel tests (see below) confirmed that the proportion of flies responding to this adsorbent collection was equivalent to a sample of the six-component synthetic blend at a comparable dose.

Behavioral Responses of Dogwood Flies. Only 2 of the 35 flies tested individually to a blank control treatment (red sphere with solvent treated septum) took flight from the release cage, and neither of these flies initiated upwind flight. Thus, as in previous tests with hawthorn flies (Nojima et al., 2003), there was no directed flight response of dogwood flies to the visual stimulus of the red sphere from the release distance of 1 m.

Experiment 1. Behavioral Responses of Dogwood Flies to an Adsorbent Collection Extract and the Synthetic Blend. A total of 44% of the dogwood flies tested reached the sphere when it contained the 50-μg source containing volatiles from whole fruit. This value was not significantly different from the 53% of flies reaching spheres containing the 50-μg source with the six-component synthetic blend (Figure 1; Fisher's exact $P = 0.655$, df $= 1$). However, more flies (85%) flew

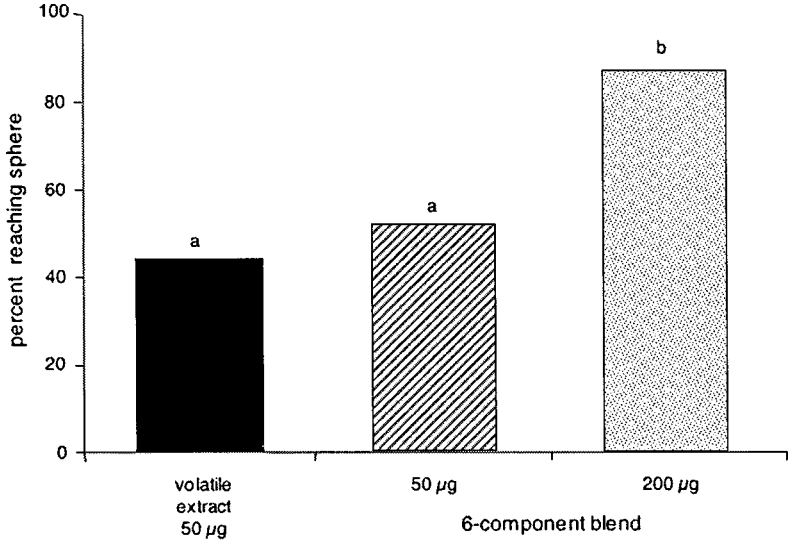


FIG. 1. The percentage of dogwood flies flying upwind and reaching spheres containing a 50-μg dose of the dogwood fruit volatile extract, and 50- and 200-μg doses of the 6-component synthetic blend of dogwood volatiles. $N = 40$ flies tested/dose. Values with different letters represent significant differences; Fisher's exact test, $P < 0.05$.

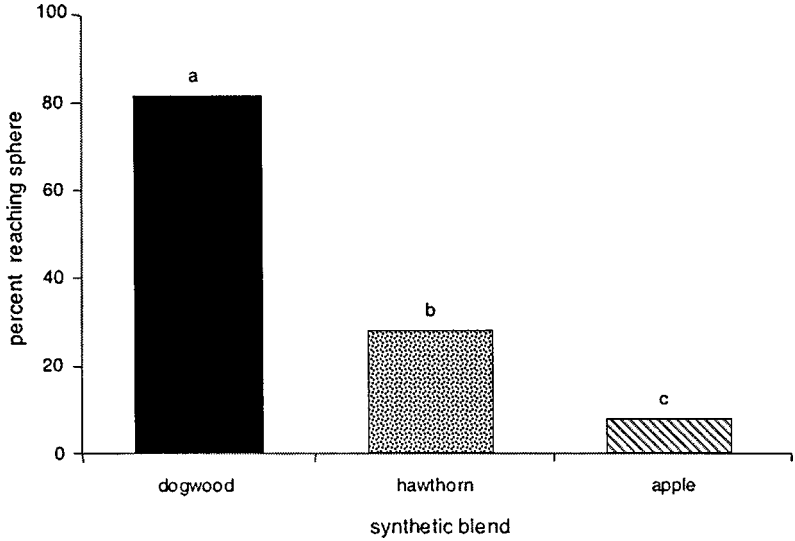
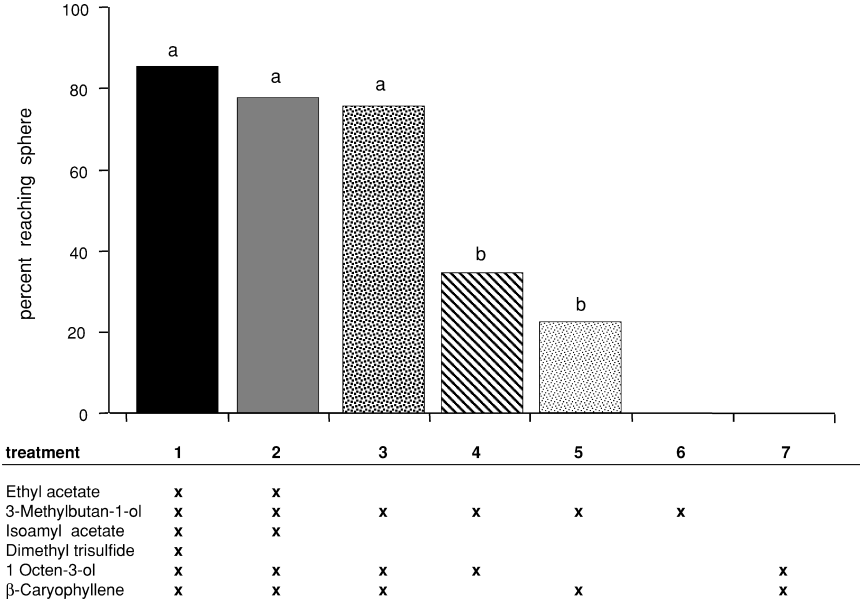


FIG. 2. Percentages of dogwood flies flying upwind and reaching spheres to a 200-μg dose of the 6-component dogwood blend, the 6-component hawthorn blend, or the 5-component apple blend (see Table 1 and methods for chemicals and proportions). $N = 42$ flies tested to dogwood blend, 49 flies hawthorn blend, and 43 flies to apple blend. Values with different letters represent significant differences; Fisher's exact test, $P < 0.05$.

upwind to a 200-μg dose of the 6-component blend, compared with the 50-μg dose of the synthetic blend (Fisher's exact $P = 0.005$, df $= 1$), or the 50-μg dose of the volatile collection (Fisher's exact $P = 0.003$, df $= 1$).

Experiment 2. Behavioral Response of Dogwood Flies to Dogwood, Hawthorn, and Apple Volatile Blends. With the 200-μg source of the six-component dogwood blend, 84% of dogwood flies reached the sphere (Figure 2). This value was greater than the 25% response value observed with the 200-μg six-component hawthorn blend (Fisher's exact $P = 0.001$, df $= 1$), and the 4% response value for the five-component apple blend (Fisher's exact $P = 0.001$, df $= 1$). Further, the 25% response value observed with the hawthorn blend was greater than the 4% for five-component apple blend (Fisher's exact $P = 0.008$, df $= 1$). The decreased response of dogwood flies to the hawthorn and apple blends was manifested at different behavioral stages in the orientation response. The lower response to the hawthorn blend primarily resulted from a decrease in the proportion of flies initiating upwind flight compared with those taking flight (74% vs. 34%; Fisher's exact $P = 0.001$, df $= 1$). In contrast, the lower proportion of flies reaching the sphere with the apple blend was mostly due to fewer flies taking flight,



treatment	1	2	3	4	5	6	7
Ethyl acetate	x	x					
3-Methylbutan-1-ol	x	x	x	x	x	x	
Isoamyl acetate	x	x					
Dimethyl trisulfide	x						
1 Octen-3-ol	x	x	x	x			x
β-Caryophyllene	x	x	x		x		x

FIG. 3. Percentage of dogwood flies flying upwind to a red sphere containing a 200-μg dose of the 6-component dogwood blend, or 6 different partial blends/single compounds. $N = 40$ flies tested/treatment for treatments 1–5, and $N = 15$ each for treatments 6 and 7. Values with different letters represent significant differences in selected 2×2 comparisons (Fisher's exact test, $P < 0.05$; see text).

compared with the hawthorn blend (73% for hawthorn vs. 23% for apple; Fisher's exact $P \leq 0.001$, df $= 1$).

Experiment 3. Behavioral Responses of Dogwood Flies to Partial Mixtures of the Synthetic Blend. There was no difference in the proportion of upwind flights by dogwood flies to a three-component mixture of 3-methylbutan-1-ol, 1-octen-3-ol, and β-caryophyllene, compared to the response observed to the full six-component blend (85% vs. 76%; treatment 3 vs. 1) (Fisher's exact $P = 0.237$, df $= 1$), or the six-component blend minus dimethyl trisulfide (78%, treatment 2; Fisher's exact $P = 0.315$, df $= 1$) (Figure 3). Responses to the two-component blends of 3-methylbutan-1-ol + 1-octen-3-ol (treatment 4) and 3-methylbutan-1-ol + β-caryophyllene (5) were not different from each other (36% and 22%; Fisher's exact $P = 0.306$ df $= 1$), but the proportion of flies reaching the sphere with the two-component blend of 3-methylbutan-1-ol + 1-octen-3-ol (treatment 4) was lower compared with the response to the six-component blend (treatment 1; Fisher's exact $P = 0.001$, df $= 1$). Finally, 3-methylbutan-1-ol (treatment 6) or 1-octen-3-ol + β-caryophyllene (7) did not elicit any upwind flight when presented as single compounds.

DISCUSSION

Using a protocol involving SPME and GC-EAD analysis previously employed for apple- and hawthorn-origin flies (Zhang et al., 1999; Nojima et al., 2003), we have identified a blend of volatile compounds emitted from flowering dogwood fruit that gave consistent EAD activity from *Rhagoletis pomonella* flies infesting dogwood fruit: ethyl acetate, isoamyl acetate, 3-methylbutan-1-ol, dimethyl trisulfide, 1-octen-3-ol, and β-caryophyllene. Adult dogwood flies responded in a flight tunnel assay at equivalent levels to 50-μg doses of an extract of whole fruit and a synthetic blend of the six chemicals. This supports the conclusion that our synthetic blend contains all of the key compounds resulting in attraction of flies to the sphere. However, based on results of subtraction assays, a three-component mix of 3-methylbutan-1-ol, 1-octen-3-ol, and β-caryophyllene also induced similar behavioral activity in flies to that of the six-component blend.

Our results add to previous findings (Zhang et al., 1999; Nojima et al., 2003) with different members of the *R. pomonella* species complex showing that adult flies detect and orient to unique mixtures of volatile odors from their natal host fruits. This behavior is likely to play an important role in the establishment and maintenance of host fidelity, the tendency of adult flies to mate on and oviposit into the same species of host fruit that they infested as larvae. Dogwood flies flew upwind and reached the red sphere in significantly greater numbers with the dogwood blend than with equivalent amounts of previously identified volatile blends from apple or hawthorn fruit (Zhang et al., 1999; Nojima et al., 2003). The dogwood blend is more similar to the hawthorn than the apple blend, with both blends sharing the key volatile 3-methylbutan-1-ol. The presence of this key volatile in the dogwood and hawthorn blends may partly explain the higher proportion of dogwood flies taking flight to the hawthorn than the apple blend. The compounds 1-octen-3-ol and β-caryophyllene were not found in hawthorn fruit, and this could account for the significant decrease in the number of upwind flights by dogwood flies to the hawthorn blend compared with the dogwood mixture. Butyl hexanoate, a key behavioral component in the apple blend for apple-origin *R. pomonella*, was identified in much smaller quantities in the hawthorn blend compared with apple (1% vs. 37% of the mixture, respectively), but was not present in volatiles from flowering dogwood. Tests of the effects of combined volatile blends, as well as effects of individual nonnatal fruit chemicals on the flight behavior of apple, hawthorn, and dogwood flies to their respective blends are currently being conducted.

Acknowledgments—We thank Stewart Berlocher, Jeff Feder, and Hattie Dambroski for shipments of fruit and flies from field locations. We also thank Harvey Reissig for use of the Geneva colony flies, Karrie Catropia, Cindy Smith, Callie Musto, and Kathy Poole for maintaining the Geneva lab colony and wild flies sent to the Geneva lab. Drs Bruce Morris and Aijun Zhang participated in preliminary analyses of dogwood fruit volatiles. Research was sponsored by NSF Grant DEB - 9977011.

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